

2002410918. PubMed ID: 12165799. TNFalpha induces acetylation of p53 but attenuates its transcriptional activation in rheumatoid synoviocytes. Nakazawa Minako; Aratani Satoko; Hatta Mitsutoki; Araya Natsumi; Daitoku Hiroaki; Kawahara Ko-Ichi; Watanabe Shin-Ichi; Nakamura Hiroshi; Yoshino Shin-Ichi; Fujii Ryouji; Fujita Hidetoshi; Fukamizu Akiyoshi; Nishioka Kusuki; Nakajima Toshihiro. (Rheumatology, Immunology and Genetics Program, Institute of Medical Science, St. Marianna University, School of Medicine, Kanagawa, Japan.) International journal of molecular medicine, (2002 Sep) Vol. 10, No. 3, pp. 269-75. Journal code: 9810955. ISSN: 1107-3756. Pub. country: Greece. Language: English.

AB Synovial hyperplasia is an important feature of rheumatoid arthritis (RA) and we have reported that several transcription factors were highly activated in rheumatoid synoviocytes. The purpose of this study was to examine nuclear acetylation in synoviocytes as an activation marker and determine its role in cell activation. Autonomous acetylation of approximately 53 and 62 kDa nuclear proteins was detected in rheumatoid synoviocytes by **anti-acetylated lysine** specific antibody. Furthermore, tumor necrosis factor alpha (TNFalpha), a potent mitogen for synoviocytes, dose-dependently increased their state of acetylation. Immunoprecipitation analysis revealed that 53 kDa acetylated protein (ap53) was identical with p53, a tumor suppressor gene product. Since enhanced p53 binding to the promoter by TNFalpha treatment was detected by gel shift assay, we analyzed p53 promoter activity by reporter assay system. Contrary to enhanced binding activity, the transcriptional activity was attenuated in a TNFalpha concentration-dependent manner. Since p53 activation requires recruitment of CREB binding protein (CBP) as a coactivator, we also examined the effect of CBP on TNFalpha-induced attenuation of p53 promoter activation. Overexpression of CBP induced p53 transcriptional activity and recovery of TNFalpha-induced inhibition. Our results clearly indicate that autonomous nuclear acetylation is characteristically enhanced in rheumatoid synoviocytes and that p53 is one of acetylated protein. Our results also demonstrate that TNFalpha-induced acetylation of p53 attenuated its transcriptional activation via CBP depletion, and that overexpression of CBP enhanced TNFalpha-induced cell death in rheumatoid synoviocytes, suggesting that regulation of transcriptional coactivator become a novel strategy for RA therapy

. protein by p300 is important for its transcriptional activity. Ott M; Schnolzer M; Garnica J; Fischle W; Emiliani S; Rackwitz H R; Verdin E. (Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, 69120, Germany.) Current biology : CB, (Dec 16-30 1999) Vol. 9, No. 24, pp. 1489-92. Journal code: 9107782. ISSN: 0960-9822. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The **human immunodeficiency virus 1 (HIV-1) Tat** protein activates transcriptional elongation by recruiting the positive transcription elongation factor (pTEFb) complex to the TAR RNA element, which is located at the 5' extremity of all viral transcripts [1-3]. **Tat** also associates in vitro and in vivo with the transcriptional coactivator p300/CBP [4-6]. This association has been proposed to recruit the histone acetyltransferase (HAT) activity of p300 to the integrated **HIV-1** promoter. We have observed that the purified p300 HAT domain acetylates recombinant **Tat** proteins in vitro and that **Tat** is acetylated in vivo. The major targets of acetylation by p300 are lysine residues (Lys50 and Lys51) in the arginine-rich motif (ARM) used by **Tat** to bind RNA and for nuclear import. Mutation of these residues in full-length recombinant **Tat** blocked its acetylation in vitro. Furthermore, mutation of these lysine residues to arginine markedly decreased the synergistic activation of the **HIV** promoter by **Tat** and p300 or by **Tat** and cyclin T1. These results demonstrate that acetylation of **Tat** by p300/CBP is important for its transcriptional activation of the **HIV** promoter.

2000012920. PubMed ID: 10545121. HIV-1 **tat** transcriptional activity is regulated by **acetylation**. Kiernan R E; Vanhulle C; Schiltz L; Adam E; Xiao H; Maudoux F; Calomme C; Burny A; Nakatani Y; Jeang K T; Benkirane M; Van Lint C. (Laboratoire de Virologie Moleculaire et Transfert de Gene, Institut de Genetique Humaine, UPR1142 Montpellier, 34396, France.) The EMBO journal, (1999 Nov 1) Vol. 18, No. 21; pp. 6106-18. Journal code: 8208664. ISSN: 0261-4189. Pub. country: ENGLAND: United Kingdom: Language: English.

AB The **human immunodeficiency virus (HIV)** trans- activator protein, **Tat**, stimulates transcription from the viral long-terminal repeats (LTR) through an RNA hairpin element, trans-activation responsive region (TAR). We and others have shown that trans-activator protein (**Tat**)-associated histone acetyltransferases (TAHs), p300 and p300/CBP-associating factor (PCAF), assist functionally in the activation of chromosomally integrated HIV-1 LTR. Here, we show that p300 and PCAF also directly acetylate **Tat**. We defined two sites of acetylation located in different functional domains of **Tat**. p300 acetylated Lys50 in the TAR RNA binding domain, while PCAF acetylated Lys28 in the activation domain of **Tat**. In support of a functional role for acetylation in vivo, histone deacetylase inhibitor (trichostatin A) synergized with **Tat** in transcriptional activation of the HIV-1 LTR. Synergism was TAR-dependent and required the intact presence of both Lys28 and Lys50. Mechanistically, acetylation at Lys28 by PCAF enhanced **Tat** binding to the **Tat**-associated kinase, CDK9/P-TEFb, while acetylation by p300 at Lys50 of **Tat** promoted the dissociation of **Tat** from TAR RNA that occurs during early transcription elongation. These data suggest that acetylation of **Tat** regulates two discrete and functionally critical steps in transcription, binding to an RNAP II CTD-kinase and release of **Tat** from TAR RNA

8 / 8

FIG. 5B

60	70	83	84	90	103
KRRQRRRPPQGSQTHQVSLSKQPTSQSRG-DPT	GPKESKKKVERETETD	PFD-			(SEQ ID NO: 42)
KRRQRRRPPQGSQTHQVSLSKQPTSQSRG-DPT	GPKE--	----			(SEQ ID NO: 43)
KRRQRRRAPQGSQTHQVSLSKQPTSQSRG-DPT	GPKE--	----			(SEQ ID NO: 44)
KRRQRRRPPQGSQTHQVSLSKQPTSQPRG-DPT	GPKE--	----			(SEQ ID NO: 45)
KRRQRRRPPQGSQTHQVSLSKQPTSQPRG-DPT	GPKE--	----			(SEQ ID NO: 46)
KRRQRRGPPQGSQTHQVSLSKQPTSQPRG-DPT	GPKESKEKVERETETD	PAVQ			(SEQ ID NO: 47)
KRRQRRRAHQNSQTHQASLSKQPTSQPRG-DPT	GPKE--	----			(SEQ ID NO: 48)
KRRQRRRAHQDSQNHQASLSKQSSQTRG-DPT	GPKEPKKEVEREAETD	PLD-			(SEQ ID NO: 49)
KRRQRRAPDSSQNHQDLSKQSSQPRG-DPT	GPKESKKEVERETETD	PLD-			(SEQ ID NO: 50)
KRRQRRRPSGGQTHQDPIPKQSSQPRG-NPT	GPKE--	----			(SEQ ID NO: 51)
KRRQRRRPSGGQTHQDPIPKQSSQPRG-DPT	GPKE--	----			(SEQ ID NO: 52)
KRRQRRGPPGGQAHQVPIPKQSSQPRG-DPT	GPKEQKKKVESEAETD	--			(SEQ ID NO: 53)
KRRQRRKPPQGDQAHQVPIPEQSSQSRG-DPT	GPKK--	----			(SEQ ID NO: 54)
KRRQRRRPPQNAHQDPLPEQSSQHRGDHPT	GPKE--	----			(SEQ ID NO: 55)
KRKPRRGPPQGSKDHTLIPKQPLPQSQR-VSA	GQEESKKKVESKAKTDR	FA-			(SEQ ID NO: 56)
KRRQRRRAPQDSQTHQASLSKQPASQSRG-DPT	GPTESKKKVERETETD	PFD-			(SEQ ID NO: 57)
KRRQRRRPPQDSQTHQSSLSKQPTSQLRG-DPT	GPTESKKKVERETETD	PVH-			(SEQ ID NO: 58)
KRRQRRRAPQDSKTHQVSLSKQPASQPRG-DPT	GPKESKKKVERETETD	PED-			(SEQ ID NO: 59)
KRRQRRRA-----	-----	----			(SEQ ID NO: 60)
KRRQRRRAPEDSQTHQVSLPKQAPQFRG-DPT	GPKESKKKVERETETH	PVD-			(SEQ ID NO: 61)
KRRQRRRAPQDSQTHQVSLPKQPASQARG-DPT	GPKESKKKVERETETD	PVD-			(SEQ ID NO: 62)
KRRQRRRAPPDSEVHQVSLPKQPASQPGQ-DPT	GPKESKKKVERETETD	PVH-			(SEQ ID NO: 63)
KRRQRRRPPQDSQTHQVSLPKQPSQQRG-DPT	GPKESKKKVERETETD	PDN-			(SEQ ID NO: 64)
KRRQRRRPPQGSQTHQVSLSKQPTSQSRG-DPT	GPKESKKKVERETETD	PFD-			(SEQ ID NO: 65)

7 / 8

FIG. 5A

Tat alignment

	1	10	20	30	40	50
AAL29460	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P04606	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P04326	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P04607	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P04610	(1) MEPVDPRLEPWKH	PGSQPKTACTT	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P05908	(1) MEPVDPRLEPWKH	PGSQPKTACNN	CYCKKCCYHC	QVCFITKGL	GISYGRK	
P04608	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P05907	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P19552	(1) MEPVDPNLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKGL	GISYGRK	
P04609	(1) MDPVDPNLEPNH	PGSQPKTACNR	CHCKKCCYHC	QVCFITKGL	GISYGRK	
P12506	(1) MDPVDPNIEPNH	PGSQPKTACNR	CHCKKCCYHC	QVCFITKGL	GISYGRK	
P04611	(1) MDPVDPNLEPNH	PGSQPRTPCNK	CHCKKCCYHCP	VCFITKNG	LGISYGRK	
P18804	(1) MDPVDPNLESWN	PGSQPRTACNK	CHCKKCCYHC	QVCFITKGL	GISYGRK	
P04613	(1) MDPVDPNLEPNH	PGSQPRTPCNK	CYCKKCCYHC	QVCFITKGL	GISYGRK	
P24738	(1) MEPVDPNLEPWKH	PGSQPTTACSN	CYCKVCCWHC	QLCFLKK	LGISYGKK	
P04614	(1) MEPVDPNLEPWKH	PGSQPRTACNN	CYCKKCCFHC	QVCFITRK	LGISYGRK	
P35965	(1) MEPVDPNLEPWKH	PGSQPRTACNN	CYCKKCCFHC	QVCFITKGL	GISYGRK	
P20893	(1) MEPVDPRLEPWKH	PGSQPKTASNN	CYCKRCCFHC	QVCFITKGL	GISYGRK	
P04612	(1) MEPVDPRLEPWKH	PGSQPKTACTT	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P05905	(1) MEPVDPRLEPWKH	PGSQPKTACTT	CYCKKCCFHC	QVCFITKAL	GISYGRK	
P05906	(1) MDPVDPRLEPWKH	PGSQPKAACT	SCYCKKCCFHC	QVCFITKGL	GISYGRK	
P19553	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKGL	GISYGRK	
P20879	(1) MEPVDPSLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKGL	GISYGRK	
Consensus	(1) MEPVDPRLEPWKH	PGSQPKTACTN	CYCKKCCFHC	QVCFITKAL	GISYGRK	

DNA AND CELL BIOLOGY
Volume 21, Number 9, 2002
© Mary Ann Liebert, Inc.
Pp. 599-610

HIV-1 Tat-Based Vaccines: From Basic Science to Clinical Trials

EMANUELE FANALES-BELASIO,¹ AURELIO CAFARO,¹ ANDREA CARA,¹
DONATELLA R.M. NEGRI,¹ VALERIA FIORELLI,¹ STEFANO BUTTO,¹ SONIA MORETTI,¹
MARIA TERESA MAGGIORELLA,¹ SILVIA BARONCELLI,¹ ZULEIKA MICHELINI,¹
ANTONELLA TRIPICIANO,¹ LEONARDO SERNICOLA,¹ ARIANNA SCOGGIO,¹
ALESSANDRA BORSETTI,¹ BARBARA RIDOLFI,¹ ROBERTA BONA,¹ PETER TEN HAAFT,²
IOLE MACCHIA,¹ PASQUALINA LEONE,¹ MARIA ROSARIA PAVONE-COSSUT,¹ FILOMENA NAPPI,¹
EFTYHIA VARDAS,³ MAURO MAGNANI,⁴ ELENA LAGUARDIA,⁴ ANTONELLA CAPUTO,⁵
FAUSTO TITTI,¹ and BARBARA ENSOLI¹

ABSTRACT

Vaccination against human immunodeficiency virus (HIV)-1 infection requires candidate antigen(s) (Ag) capable of inducing an effective, broad, and long-lasting immune response against HIV-1 despite mutation events leading to differences in virus clades. The HIV-1 Tat protein is more conserved than envelope proteins, is essential in the virus life cycle and is expressed very early upon virus entry. In addition, both humoral and cellular responses to Tat have been reported to correlate with a delayed progression to disease in both humans and monkeys. This suggested that Tat is an optimal target for vaccine development aimed at controlling virus replication and blocking disease onset. Here are reviewed the results of our studies including the effects of the Tat protein on monocyte-derived dendritic cells (MDDCs) that are key antigen-presenting cells (APCs), and the results from vaccination trials with both the Tat protein or *tat* DNA in monkeys. We provide evidence that the HIV-1 Tat protein is very efficiently taken up by MDDCs and promotes T helper (Th)-1 type immune responses against itself as well as other Ag. In addition, a Tat-based vaccine elicits an immune response capable of controlling primary infection of monkeys with the pathogenic SHIV89.6P at its early stages allowing the containment of virus spread. Based on these results and on data of Tat conservation and immune cross-recognition in field isolates from different clades, phase I clinical trials are being initiated in Italy for both preventive and therapeutic vaccination.

INTRODUCTION

HIV INFECTION IS PROGRESSIVELY SPREADING, particularly in the developing countries where the increasing number of deaths due to AIDS strongly and urgently calls for an effective, safe, and inexpensive vaccine against AIDS. Since the characterization of HIV in the early 80s, many attempts have been made to find and develop a candidate vaccine of proven safety

and efficacy. However, due to the high variability of the envelope (Env) proteins and to the limited accessibility of relevant cross-neutralizing epitopes, vaccine studies aimed at inducing adequate titers of long-lasting antibodies (Ab) neutralizing different virus strains have substantially failed (Parren *et al.*, 1999). Nevertheless, protection against heterologous pathogenic viruses was achieved in nonhuman primates vaccinated with live-attenuated simian immunodeficiency virus (SIV), pro-

¹Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy.

²Department of Virology, Biomedical Primate Research Centre, Rijswijk, The Netherlands.

³Chris Hani Baragwanath Hospital and University of the Witwatersrand, South Africa.

⁴Institute of Biological Chemistry "Giorgio Fornaini," University of Urbino, 61029 Urbino, Italy.

⁵Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy.

viding the first evidence that protective immunity can be induced and a valuable tool to better understand the mechanisms and correlates of protection from pathogenic viruses (for a review, see Ensoli and Cafaro, 2001). However, the appearance of revertant pathogenic viruses and the apparent pathogenicity of the attenuated viruses in newborn macaques (Baba *et al.*, 1995; Whatmore *et al.*, 1995; Ezzel, 2002) constitute at the present an insurmountable obstacle to their use for vaccination in humans.

Because Env-based vaccines have failed at inducing a sterilizing immunity against different virus strains, secondary end points are being considered in vaccine strategies, such as the control of viral infection and the block of disease onset. To this goal, different vaccine approaches, based on recombinant proteins, DNA plasmids or expression vectors (mostly in prime-boost regimens) are being tested in animal models or human volunteers. Interest has also recently focused towards those non-structural viral proteins that have a key role in the virus life cycle and are adequately immunogenic and conserved among several virus subtypes. Among these, the HIV-1 Tat protein fulfills these requirements and, to date, is considered as a potential and promising vaccine candidate (for a review, see Ensoli and Cafaro, 2001). The more attractive features of Tat are: (1) its early expression and critical role in the virus life cycle; (2) the correlation of the anti-Tat immune response with nonprogression to AIDS in infected individuals; (3) its peculiar property to be efficiently taken up by professional antigen presenting cells (APCs) and to be presented in the context of the major histocompatibility complex (MHC) class I; (4) the conservation among geographically distinct isolates; (5) the safety, immunogenicity, and efficacy of Tat vaccination in macaques. These aspects are briefly described.

Role of Tat in the virus life cycle

The Tat protein of HIV is expressed very early after infection before the structural genes (*env*, *gag*, and *pol*), even before viral integration (Wu and Marsh, 2001). Tat is a potent transcriptional transactivator of HIV-1 gene expression (Arya *et al.*, 1985; Fisher *et al.*, 1986), and essential for viral replication, transmission, and disease progression (H.K. Chang *et al.*, 1994, 1995). In the absence of Tat, in fact, extremely poor amounts of structural proteins are expressed with consequent negligible production of infectious virus (Dayton *et al.*, 1986; Fisher *et al.*, 1986). Further, Tat is released by infected T lymphocytes in the extracellular milieu (Ensoli *et al.*, 1990, 1993; H.C. Chang *et al.*, 1997), and can enter both infected cells, in which promotes HIV replication, and uninfected cells in which induces the expression or repression of cellular genes controlling the cell cycle and cell activation (Frankel and Pabo, 1988; Ensoli *et al.*, 1993; H.K. Chang *et al.*, 1994, 1995; Li *et al.*, 1997). In addition, Tat has been recently shown to induce the expression of the chemokine receptors (and HIV-1 coreceptors) CCR5 and CXCR4 (Huang *et al.*, 1998; Secchiero *et al.*, 1999), essential for the infectivity of macrophage- and T-cell-tropic HIV-1 strains, respectively. Of note, because infected cells express Tat very early after infection, they may represent a relevant target for cytotoxic T lymphocytes (CTLs) that may block infection at

its early stages. Therefore, an effective immune response to Tat may efficiently inhibit virus replication and dissemination, thus preventing the progression to AIDS.

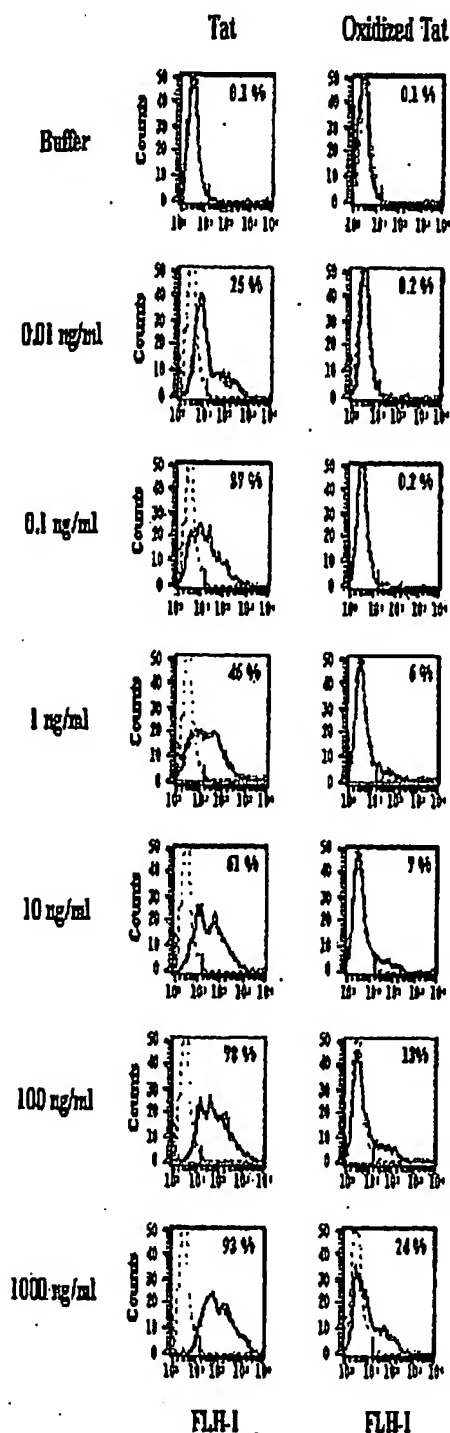
The protective role of the anti-Tat immune response

Several studies support the concept that an immune response to Tat has a protective role, and may control disease progression *in vivo*. First, the protein is immunogenic and well conserved (see the following section), eliciting Ab responses that have been associated with nonprogression to AIDS (Reiss *et al.*, 1990; Rodman *et al.*, 1993; Re *et al.*, 1995; Zagury *et al.*, 1998). Accordingly, by using an algorithm based on two optimized ELISA assays, we have observed a higher prevalence of both anti-Tat IgM and IgG Ab in asymptomatic Italian patients compared to patients in advanced stages of disease (S. Buttò, V. Fiorelli, A. Tripiciano, *et al.*, in preparation). Similarly, CTLs to Tat are frequently detected in asymptomatic HIV-1-infected individuals (Addo *et al.*, 2001), and have been shown to inversely correlate with progression to AIDS (van Baalen *et al.*, 1997). This correlation has been recently confirmed by us in vaccine trials with nonhuman primates (Cafaro *et al.*, 1999, 2000, 2001). Finally, a recent study (Allen *et al.*, 2000) has shown that anti-Tat CTLs are key to control early virus replication during primary SIV infection, exerting an immune pressure leading to selection of less pathogenic escape mutants.

Uptake, presentation, and induction of Th-1 responses by soluble native Tat protein

Extracellular Tat is taken up by cells (Frankel and Pabo, 1988; Ensoli *et al.*, 1993; H.C. Chang *et al.*, 1997; Tyagi *et al.*, 2001), and, unlike most soluble proteins, enters the MHC class I pathway of presentation and elicits CTL activity (Moy *et al.*, 1996; Kim *et al.*, 1997), a feature that Tat shares with very few other soluble proteins called "penetratins" (reviewed by Derossi *et al.*, 1998).

We investigated further these aspects and found that monocyte-derived dendritic cells (MDDCs), among the most potent APCs, take up Tat much more efficiently than other cell types such as T cell blasts and B-lymphoblastoid cell lines and in a time-, dose- and maturation-dependent fashion (Fanales-Belasio *et al.*, 2002). Specifically, Tat is taken up by MDDCs at doses as low as 0.01 ng/ml (Fig. 1) and the uptake peaks after 5–10 min. This process is markedly hampered by the oxidation/inactivation of the protein (Fig. 1), and by low temperature. Of note, the uptake is more efficient upon MDDCs maturation, suggesting the involvement of a receptor-mediated pathway upregulated upon cell maturation, and excluding an involvement of the pinocytotic pathways, both diminished in mature MDCCs (Bell *et al.*, 1999). Moreover, Tat also induces MDCCs maturation at relatively high doses (1.2 to 20 µg/ml), as indicated by a dose-dependent increase of both the surface expression of MHC and costimulatory (CD40, CD80, CD86) molecules (Fig. 2A) and of the production of interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) and of the β-chemokines MIP-1α, MIP-1β, and RANTES (Fig. 2B). In contrast, oxidized Tat had no effects. Consistent with these data, MDDCs treated with Tat have an increased capacity to present both allogeneic and recall Ag potentiating



T cell responses against heterologous Ags (Fig. 3) (Fanales-Belasio *et al.*, 2002).

Conservation of Tat among geographically distinct isolates

Studies conducted in Uganda and in South Africa indicate that the Tat sequence, from African individuals infected with A, C, and D subtypes, is conserved in its immunogenic epitopes (Buttò *et al.*, in preparation) and that sera from these individuals recognize and neutralize *in vitro* the activity of a Tat protein derived from a distantly related subtype B isolate (Buttò *et al.*, in preparation). Furthermore, a recent sequence data analysis confirmed the conservation of the immunodominant B cell epitopes of Tat among distantly related HIV-1 subtypes (Goldstein *et al.*, 2001; and our unpublished data). Thus, there is clear evidence that, due to cross-clade recognition, a Tat-based approach may work against infection with different viral clades.

Safety, immunogenicity, and efficacy of anti-Tat vaccine in macaques

Injection of biologically active Tat protein or wild-type *tat* DNA is safe, as indicated by our safety studies conducted in 276 mice, 48 guinea pigs, and 27 monkeys in which no local or systemic toxicity or adverse effects (at the biochemical, hematologic, or immunologic level) were ever observed (Caselli *et al.*, 1999; Cafaro *et al.*, 2001; our unpublished data). Data from others confirmed the absence of toxicity of biologically active Tat or *tat* DNA in mice or monkeys (Hinkula *et al.*, 1997; Osterhaus *et al.*, 1999; Pauza *et al.*, 2000; Nilsson *et al.*, 2001; Allen *et al.*, 2002; Silvera *et al.*, 2002; Caputo *et al.*, 2002, in press). In addition, no enhancement of virus replication nor CD4+ T cell decline was detected in SHIV89.6P-infected monkeys with AIDS that received Tat protein and *tat* DNA in a safety study of the therapeutic approach (our unpublished data). Moreover, vaccination of HIV-1-infected individuals with *tat* DNA confirmed that immunization with Tat is safe, because no induction of virus transcription was observed, despite the evidence of boosting of specific immune responses (Calarota *et al.*, 1998, 1999).

The immunogenicity of the HIV-1 Tat protein or *tat* DNA has also been demonstrated in mice and in monkeys in which vaccination with the Tat protein or *tat* DNA induced both humoral and cellular (including CTL activity) Tat-specific immune responses (Hinkula *et al.*, 1997; Caselli *et al.*, 1999; Ca-

FIG. 1. Native, but not oxidized, Tat is efficiently taken up by MDDCs. Human MDDCs were incubated with serial concentrations (0.01 to 1,000 ng/ml) of the HIV-1 Tat protein, native or oxidized by exposure to light and air for 16 h, or Tat reconstitution buffer. After 10 min cells were washed, fixed, permeabilized, stained with a specific affinity-purified rabbit anti-Tat polyclonal Ab (or isotype control) followed by secondary FITC-conjugated antirabbit Ab, and analyzed by flow cytometry as reported (Fanales-Belasio *et al.*, 2002). The percentage of positive cells (compared to isotype stained samples) is reported in each panel. Data are from one donor representative of the 14 analyzed.

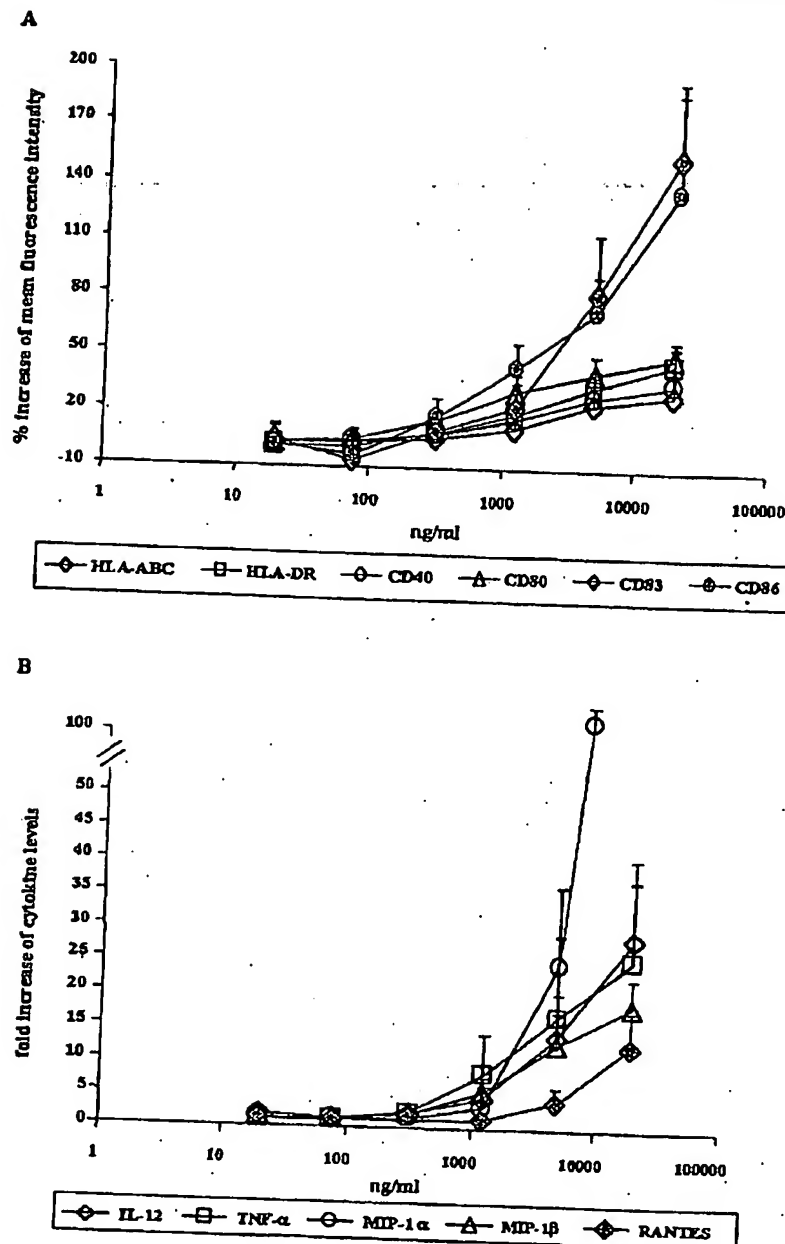


FIG. 2. Tat enhances both the expression of surface HLA and costimulatory molecules and the production of the cytokines IL-12 and TNF- α and of the β -chemokines MIP-1 α , MIP-1 β , and RANTES in human MDDCs. (A) Cells from 10 different donors were exposed for 18 h to native Tat (20 to 20,000 ng/ml) or reconstitution buffer, stained with fluorochrome-conjugated monoclonal Ab, and then analyzed by flow cytometry. The mean (and SEM) percentage increases of the expression (as mean fluorescence intensity) of HLA-ABC (diamonds), HLA-DR (squares), CD40 (circles), CD80 (triangles), CD83 (gray diamonds), and CD86 (gray circles) are reported. (B) Supernatants of MDDCs exposed for 18 h to Tat (20 to 20,000 ng/ml) or reconstitution buffer were assayed by ELISA to measure the levels of IL-12 (diamonds), TNF- α (squares), MIP-1 α (circles), MIP-1 β (triangles), and RANTES (gray diamonds). The mean (and SEM) fold increases of the cytokine levels (pg/ml) induced by Tat compared to buffer are reported. A very poor cytokine or β -chemokine production was induced by oxidized-inactivated Tat protein (E. Fanales-Belasio, S. Moretti, G. Barillari, *et al.*, unpublished data).

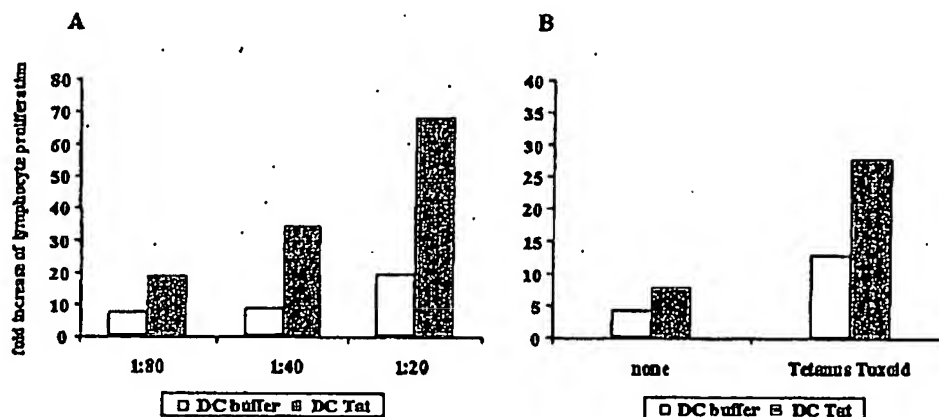


FIG. 3. Tat enhances both allogeneic and recall antigen presentation by MDDCs. (A) Cells were exposed for 18 h to native Tat (10,000 ng/ml) (gray columns) or reconstitution buffer (white columns) and cultured together with allogeneic peripheral blood lymphocytes (PBLs) at different cell to cell ratios. ^3H -thymidine uptake was measured after 6 days of culture to evaluate lymphocyte proliferation. Data from a representative experiment out of four performed with different donors are expressed as the fold increase of lymphocyte proliferation at the MDDCs/PBLs ratios of 1:20, 1:40, and 1:80. (B) MDDCs exposed to Tat, as above reported, were cultured in the presence or absence of tetanus toxoid (TT 5 $\mu\text{g}/\text{ml}$) together with syngeneic PBLs (ratio 1:20) from a patient responsive to this recall Ag. Data are expressed as the fold increase of lymphocyte proliferation.

faro *et al.*, 1999, 2000, 2001; and unpublished data; Allen *et al.*, 2002; Silvera *et al.*, 2002; Caputo *et al.*, 2002). Similar results were reported in monkeys immunized with viral vectors expressing SIV Tat and Rev (Osterhaus *et al.*, 1999) or Env, Gag-Pol, Nef, Rev, and Tat (Nilsson *et al.*, 2001). In addition, an increase in humoral and cellular anti-Tat immune responses was detected in HIV-1 infected individuals immunized with HIV-1 *tat* DNA (Calarota *et al.*, 1998, 1999). Finally, our recent data indicate that in mice the mucosal or systemic administration of a biologically active Tat protein alone or combined with an unrelated Ag (Candida, ovalbumin, streptavidin) is safe, and elicits immunity to both (M. Marinaro, A. Ricconi, R. Rappuoli, *et al.*, submitted; S. Bort-sutzky, V. Fiorelli, F. Rharbaoui, *et al.*, in preparation; S. Dom-inici, M.E. Laguardia, G. Serafini, *et al.*, in preparation).

To evaluate the efficacy of a preventive *tat* vaccine in controlling virus replication, preclinical trials with the Tat protein

or *tat* DNA were carried out in monkeys as two arms of the same protocol.

In the Tat protein protocol (Cafaro *et al.*, 1999, 2000), 7 *Macaca fascicularis* (cynomolgus monkeys) were immunized with a biologically active Tat protein, as reported in Table 1. Six monkeys were immunized subcutaneously (s.c.) with 10 μg of Tat with RIBI (three monkeys) or Alum (three monkeys) as adjuvant, while one received Tat alone (6 μg) intradermally (i.d.). Two control monkeys were injected s.c. with either RIBI or Alum alone, and two naive monkeys were included at the challenge as additional controls. The six monkeys vaccinated s.c. received eight boosts in 36 weeks, while a ninth final boost was performed at week 42 intramuscularly (i.m.) with Tat associated to immune stimulating complexes (ISCOMs) (Davis *et al.*, 1997). The monkey immunized i.d. was boosted nine times, and did not receive the ISCOMs boost.

TABLE 1. TAT PROTEIN VACCINE PROTOCOL

Monkey	Immunogen	Adjuvant	Administration
54844 54879 54963	Tat protein (10 $\mu\text{g}/250$ ml)	RIBI (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)
54899 55396 54240	Tat protein (10 $\mu\text{g}/250$ ml)	Alum (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)
54222	Tat protein (6 $\mu\text{g}/\text{ml}$)	Nil	Intradermic, 150 μl in two sites (upper dorsal area)
55123	Saline buffer (250 μl)	RIBI (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)
55129	Saline buffer (250 μl)	Alum (250 μl)	Subcute, 500 μl in one site (dorsal area, close to the neck)

For further details refer to Cafaro *et al.*, 1999.

No signs or symptoms related to toxicity (acute or chronic, local or systemic) were ever detected in both vaccinated and control animals throughout the immunization period.

The six monkeys inoculated with Tat and RIBI or Alum developed high titers of anti-Tat Ab (up to 1:25,600), capable of neutralizing the activity of Tat to rescue Tat-defective proviruses (Barillari *et al.*, 1992; Ensoli *et al.*, 1993; H.C. Cheng *et al.*, 1997). In contrast, the animal given Tat i.d. developed low and transient titers of anti-Tat Ab (1:100) (Table 2).

The monkeys immunized s.c. but not the one vaccinated i.d. developed anti-Tat T cell responses, as indicated by the presence of delayed-type hypersensitivity (DTH) and proliferative responses to Tat (Table 2). Further, a specific anti-Tat CTL activity, barely detectable in the vaccinated animals since week 28 after immunization, at week 36 reached levels above the cut-off in one out of two macaques vaccinated with Tat and RIBI, in two out of three monkeys vaccinated with Tat and Alum, and in the monkey vaccinated i.d. with Tat alone (Table 2). In addition, *in vitro* TNF- α production in response to Tat, a marker for CTLs, was found at week 44 after immunization in vaccinated and protected animals, but not in the infected ones.

At week 50 after immunization (14–18 weeks after the last boost), all the animals were challenged intravenously (i.v.) with the SHIV89.6P, a chimeric virus containing the *env* and *tat* genes of HIV-1 in the backbone of SIVmac239, and highly pathogenic in macaques (Reimann *et al.*, 1996; Karlsson *et al.*, 1997; Cafaro

et al., 2000). The virus stock used for the challenge derived from a cynomolgus macaque inoculated with the SHIV89.6P originally derived from rhesus monkeys. Also, this virus stock was highly pathogenic, inducing the death of four out of seven infected monkeys (Cafaro *et al.*, 2000; M.T. Maggiorella, S. Baroncelli, Z. Michelini, *et al.*, submitted). All animals were inoculated with 10 MID₅₀ of the virus, except the two naive control monkeys that were challenged with either a threefold lower (2.8 MID₅₀) or higher (28 MID₅₀) doses, respectively (Table 3). Of note, the macaque infected with 28 MID₅₀ was euthanized at week 35 after the viral challenge, due to severe worsening of the clinical conditions associated to extremely low CD4+ T cell counts.

After challenge, all the controls but only two out of the seven Tat protein-vaccinated monkeys (one given Tat and RIBI and one Tat and alum) were infected, as indicated by the presence of high plasma levels of p27 Ag (detected by ELISA) and viral RNA [detected by branched DNA (bDNA) and quantitative-competitive RNA-PCR (QT-RNA-PCR) assays], proviral DNA copies, cytoviremia, or positive virus isolation from PBMCs (Table 3). In contrast, all these parameters were always negative for all the other five vaccines up to 2 years postchallenge, although few copies of SIV proviral DNA (<10 copies/ μ g of DNA) were sporadically detected (Table 3). This, together with the finding of low and transient anti-SIV (or anti-HIV Env) Ab titers in the protected animals, indicated the occurrence of a very limited viral replication.

TABLE 2. TAT PROTEIN VACCINE: IMMUNOLOGICAL RESPONSES AT PRE-CHALLENGE TIME

Monkey	Vaccination	Ab titers ^a	Tat neutralization ^b	Proliferative response ^c	DTH ^d	CTL response ^e	Tat-induced TNF- α ^f
54844	RIBI + Tat (10 μ g, SC)	++	+	+	—	+	+
54879		++	++	+	+	ND	+
54963		++	++	+++	±	—	ND
54899	Alum + Tat (10 μ g, SC)	+++	+++	+++	++	+	+
55396		+++	+++	+	++	—	—
54240		+++	+++	+	±	+	+
54222	Tat protein (6 μ g, ID)	±	ND	—	—	+	+
55123	Control RIBI	—	ND	—	ND	—	—
55129	Control Alum	—	ND	—	ND	—	—

^aAnti-Tat Ab in plasma from vaccinated or control monkeys as determined by ELISA. On the basis of reciprocal of the last positive dilution (cut-off: mean of preimmune sera + 3 SD) the following ranges of positivity are defined: ±, <100; +, 100–1,000; ++, 1,000–20,000; +++, >20,000.

^bNeutralization of the Tat-mediated (60–500 ng/ml) rescue of *tat*-defective HIV-1 provirus replication in HLM-1 cells by sera from vaccinated or control monkeys. Neutralization was defined as a >50% reduction of p24 values (cut-off = 20 pg/ml) the addition of increasing amounts of Tat compared to controls. The following scoring was defined on the basis of the highest dose of Tat (ng/ml) neutralized: +, 60–120; ++, 240; +++, 500.

^cSpecific immune response to Tat as determined by proliferation assay with monkeys PBMCs. Positivity was defined on the basis of the ratio between Tat-specific and control counts: — <3; +, 3–10; ++, 11–30; +++, >30.

^dDTH response to the Tat protein in immunized monkeys. Positivity was defined on the basis of evident signs of inflammation (erythema, induration) at the site of inoculation after 48 h: —, erythema <1 mm; ±, erythema \geq 1 mm without induration; +, induration with erythema 1–4 mm; ++, induration with erythema \geq 5 mm.

^eSpecific anti-Tat CTL response detected with PBMCs after 2 weeks of *in vitro* expansion with Tat. Values above or below the cut-off (5% Tat-specific lysis) were indicated as positive (+) or negative (—), respectively.

^fTat-induced TNF- α production by PBMCs, was detected by ELISA: values above or below the cut-off (15.6 pg/ml) were indicated as positive (+) or negative (—), respectively.

For further technical details refer to Cafaro *et al.*, 1999.

EFFICACY OF HIV-1 TAT-BASED VACCINATION TRIALS

605

TABLE 3. TAT PROTEIN VACCINE: RESULTS AFTER CHALLENGE WITH THE SHIV89.6P, IV

Monkey	Vaccination	Challenge dose	Postchallenge (up to 2 years)					
			p27 ^a	Plasma viremia ^b	DNA PCR ^c	Cytoviremia or virus isolation ^d	Anti-SIV Ab ^e	CD4+ T cells ^f
54844	RIBI + Tat (10 µg, SC)	10 MID ₅₀	—	—	— (+)*	—	±	=
54879		10 MID ₅₀	—	—	— (+)*	—	±	=
54963		10 MID ₅₀	+	+	+	+	+	↓
54899	Alum + Tat (10 µg, SC)	10 MID ₅₀	—	—	— (+)*	—	±	=
55396		10 MID ₅₀	+	+	+	+	±	↓
54240		10 MID ₅₀	—	—	— (+)*	—	±	=
54222	Tat (6 µg, ID)	10 MID ₅₀	—	—	— (+)*	—	±	=
55123	Control RIBI	10 MID ₅₀	+	+	+	+	+	↓
55129	Control Alum	10 MID ₅₀	+	+	+	+	+	↓
12	Nil	2.8 MID ₅₀	+	+	+	+	+	↓
2	Nil	2.8 MID ₅₀	+	+	+	+	+	↓

^ap27 in plasma from virus-challenged monkeys as determined by ELISA. Values above or below the cut-off value (20 pg/ml) were expressed as positive (+) or negative (—), respectively.

^bPlasma-associated virus load (RNA equivalent/ml) as determined by bDNA (cut-off: 1,500 RNA copies/ml) until week 14 post-challenge, by bDNA and QT-RNA-PCR (cut-off: 50 RNA copies/ml) between week 18 and 28 post-challenge, and only by QT-RNA-PCR since week 35. Results above or below the cut-off values were expressed as positive (+) or negative (—), respectively.

^cProviral DNA copies/µg of DNA as determined by semiquantitative DNA PCR analysis. +, proviral copy number >1;

^dSHIV89.6P virus isolation from monkeys PBMCs depleted of CD8+ cells and cocultured with CEMx174. Positively was determined by p27 ELISA (cut-off = 20 pg/ml).

^ePlasma anti-SIV Ab was determined by ELISA; levels above, equal, or below the cut-off value (mean of negative control plus 3 SD) were expressed as positive (+), border line (±), or negative (—), respectively.

^fPeripheral blood counts of CD4+ cells as evaluated by flow cytometry. The symbols ↓ and =, respectively, indicate decrease (>50% decline as compared to pre-challenge values) or normality of CD4 T cell numbers.

* (+) positive DNA PCR was detected only sporadically and at low copy number (<10/µg of DNA).

For further technical details refer to Cafaro *et al.*, 1999, 2000.

Plasma levels of anti-SIV or anti-HIV Env Ab in all infected animals were high, correlating with the parameters of viremia (Table 3, and data not shown). Of note, titers in the two infected and vaccinated animals were at least 1 log lower than in the control macaques and delayed, suggesting a possible effect of vaccination also in the infected animals.

Blood CD4+ T cells counts reflected the outcome of the challenge. In the five protected monkeys they remained in the normal range during the 2 years of follow-up, whereas markedly decreased in all the controls and in the two vaccinated and infected animals (Table 3).

Notably, protection from challenge correlated (100%) with the presence, before the challenge, of anti-Tat specific CTL activity and with Tat-induced TNF-α production, but not with the presence of anti-Tat Ab.

In the *tat* DNA vaccination study (Table 4 and Cafaro *et al.*, 2001), five monkeys were immunized with a vector expressing *tat* DNA under the control of the major adenoviral late protein promoter (pCV-*tat*) (Arya *et al.*, 1985; Ensoli *et al.*, 1993), shown to induce a high expression and release of the Tat protein in the absence of cell death (Ensoli *et al.*, 1990, 1993; H.C. Chang *et al.*, 1997). Further, the pCV vector is rich in un-

methyated CpG sequences (Cafaro *et al.*, 2001), known to enhance the innate and adaptive immune responses by inducing the maturation of dendritic cells and their production of cytokines driving Th-1 responses (reviewed in Klinman *et al.*, 1999).

As shown in Table 4, four monkeys were immunized i.m. with 1 mg (three animals) or 0.5 mg (one animal) of pCV-*tat*. The fifth monkey was immunized i.d. with 0.2 mg of pCV-*tat*. One monkey was injected i.m. with the DNA vector alone (pCV-0), to evaluate the impact of its immunostimulating effects on nonspecific protection. Vaccines and the control were boosted seven times over 36–42 weeks, and the last boost was performed, except for the one immunized with pCV-0, with the Tat protein in ISCOMs. Neither local nor systemic side effects were observed in all inoculated animals, and all the hematologic parameters (blood cell counts, blood chemistry, and FACS analysis) were always in the normal range throughout the vaccination schedule and up to the time of challenge.

Vaccination with *tat* DNA was immunogenic. Anti-Tat Ab were transient and at low titers in the four animals vaccinated i.m. with pCV-*tat*, while in the monkey inoculated i.d. with 0.2 mg of pCV-*tat* titers could be detected (up to 1:1,600) through-

TABLE 4. *TAT* DNA VACCINE PROTOCOL

Monkey	Immunogen	Adjuvant	Administration
54920 55122 55361	<i>tat</i> DNA (1 mg)	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine + 0.1% methyl paraben	Intramuscular, 500 μ l in 2 sites (femoral quadriceps)
PR2	<i>tat</i> DNA (0.5 mg)	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine + 0.1% methyl paraben	Intramuscular, 400 μ l in 2 sites (femoral quadriceps)
37	<i>tat</i> DNA (1 mg)	Nil	Intramuscular, 150 μ l in 2 sites (dorsal area)
54219 55361	DNA vector (1 mg)	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine + 0.1% methyl paraben	Intramuscular, 500 μ l in 2 sites (femoral quadriceps)
55123	Saline buffer (250 μ l)	RIBI (250 μ l)	Subcutaneous, 500 μ l in one site (dorsal area)
55129	Saline buffer (250 μ l)	Alum (250 μ l)	Subcutaneous, 500 μ l in one site (dorsal area)

For further technical details refer to Cafaro *et al.*, 2001.

out the follow-up period, with a weak Tat neutralizing activity (Table 5). Anti-Tat Ab were never found in the animal inoculated with pCV-0, or in the control animals.

A proliferative response to Tat was detected in three out of four monkeys inoculated i.m. with pCV-*tat*, and in the one in-

oculated i.d., which also showed a cutaneous DTH response to the protein (Table 5), but not in the control animals.

Anti-Tat CTL activity was detected in the four monkeys vaccinated i.m. with pCV-*tat* but not in the one vaccinated i.d. or in the control animals (Table 5).

TABLE 5. *TAT* DNA VACCINE: IMMUNOLOGIC RESPONSES AT PRE-CHALLENGE

Monkey	Vaccination	Ab titers ^a	Tat neutralization ^b	Proliferative response ^c	DTH ^d	CTL response ^e
54920 55122 55361	<i>tat</i> DNA (1 mg, IM)	\pm \pm -	- ND -	+ + +	- - -	+ + +
PR2	<i>tat</i> DNA (0.5 mg, IM)	\pm	-	-	-	+
37	<i>tat</i> DNA (0.2 mg, ID)	++	+	+	+	-
54219	DNA vector (1 mg, IM)	-	-	-	ND	-
55123	RIBI alone	-	-	-	ND	-
55129	Alum alone	-	-	-	ND	-

^aAnti-Tat Ab in plasma from vaccinated or control monkeys as determined by ELISA. On the basis of reciprocal of the last positive dilution (cut-off: mean of preimmune sera + 3 SD) the following ranges of positivity are defined: \pm , <100; +, 100-1,000; ++, 1,000-20,000; +++, >20,000.

^bNeutralization of the Tat-mediated (30-500 ng/ml) rescue of *tat*-defective HIV-1 provirus replication in HLM-1 cells by sera from vaccinated or control monkeys. Neutralization was defined as a >50% reduction of p24 values (cut-off = 20 pg/ml) upon the addition of increasing amounts of Tat compared to controls. The following scoring was defined on the basis of the highest dose of Tat (ng/ml) neutralized: +, 30-120; ++, 240; +++, 500.

^cSpecific immune response to Tat as determined by proliferation assay with PBMCs from monkeys. Positivity was defined on the basis of the ratio between Tat-specific and control counts: - <3; +, 3-10; ++, 11-30; +++, >30.

^dDTH response to the Tat protein in immunized monkeys. Positivity was defined on the basis of evident signs of inflammation (erythema, induration) at the site of inoculation after 48 h: -, erythema <1 mm; \pm , erythema \geq 1 mm without induration; +, induration with erythema 1-4 mm; ++, induration with erythema \geq 5 mm.

^eSpecific anti-Tat CTL response detected with PBMCs after 2 weeks of *in vitro* expansion with the Tat Ag. Values above or below the cut-off (10% Tat-specific lysis) were indicated as positive (+) or negative (-), respectively.

For further technical details refer to Cafaro *et al.*, 2001.

EFFICACY OF HIV-1 TAT-BASED VACCINATION TRIALS

607

All animals, together with those from the Tat protein vaccination protocol, were challenged 14–18 weeks after the last boost with 10 MID₅₀ of SHIV89.6P i.v. In the weeks following the challenge and during the entire follow-up, all the macaques vaccinated IM with pCV-*tat* were negative for p27 antigenemia, plasma viremia, and virus isolation (Table 6). SIV proviral DNA was only sporadically detected at very low copy number (range 1–8 copies/μg DNA). In contrast, all the controls and the monkey vaccinated i.d. with 0.2 mg of pCV-*tat* had detectable plasma levels of both p27 and viral RNA, with repeated isolation of infectious virus from the peripheral blood, and the proviral DNA, high early after the challenge, remained detectable for the entire follow-up period (Table 6). Of note, the monkey inoculated with pCV-0 was negative for both antigenemia and plasma viremia. However, virus was isolated 18 weeks after challenge and proviral DNA (55 copies) was detected at week 14 after challenge indicating a partial protection, conceivably conferred by natural immunity.

In the three macaques vaccinated i.m. with 1 mg of pCV-*tat* and in the one injected with pCV-0 the titers of anti-SIV Ab were below the limit of detection, while in the one vaccinated i.m. with pCV-*tat* (0.5 mg) were low (range 1:2–1:100) and

transient. Conversely, they were high (over 1:1000) and stable, since week 10 after challenge, in the monkey vaccinated i.d. with pCV-*tat*, and in the controls (Table 6). Anti-SIV Ab in the naive control monkey infected with 28.5 MID₅₀ were detected at very low titers (range 1:20–1:50) only at week 29 and 35, a relatively uncommon feature that correlates with fast progression (Dykhuizen *et al.*, 1998), although anti-HIV Ab could be detected since week 10 postchallenge (Cafaro *et al.*, 2001).

In the monkeys injected i.m. with pCV-*tat* or pCV-0, in which viral parameters were mostly negative (Table 6), the number of CD4+ T cells remained in the normal range after the viral challenge and during all the follow-up period. In contrast, in the infected animals, CD4+ T cell number sharply decreased after the challenge, remaining persistently below the baseline values during the follow up. The naive control monkey infected with 28.5 MID₅₀ showed a progressive and severe CD4+ T cells decline and had to be euthanized.

Thus, like in the vaccination protocol with the Tat protein, immunization with *tat* DNA was safe, and induced an immune response to the protein, mainly cell mediated, capable of blocking virus replication to undetectable levels, thus preventing the CD4+ T cell decline and disease onset. Protection from virus

TABLE 6. TAT DNA VACCINE: RESULTS AFTER CHALLENGE WITH THE SHIV 89.6P, IV

Monkey	Vaccination	Challenge (dose)	Postchallenge (up to 2 years)					
			p27 ^a	Plasma viremia ^b	DNA PCR ^c	Cytoviremia or virus isolation ^d	Anti-SIV Ab ^e	CD4+ T cells ^f
54920	<i>tat</i> DNA (1 mg, IM)	10 MID ₅₀	–	–	– (+)*	–	–	=
55122		10 MID ₅₀	–	–	– (+)*	–	–	=
55361		10 MID ₅₀	–	–	– (+)*	–	±	=
PR2	<i>tat</i> DNA (0.5 mg, IM)	10 MID ₅₀	–	–	– (+)*	–	±	=
37	<i>tat</i> DNA (0.2 mg, ID)	10 MID ₅₀	+	+	+	+	+	↓
54219	DNA vector (1 mg, IM)	10 MID ₅₀	–	–	+	+	–	=
55123	RIBI alone	10 MID ₅₀	+	+	+	+	+	↓
55129	Alum alone	10 MID ₅₀	+	+	+	+	+	↓
12	Nil	2.8 MID ₅₀	+	+	+	+	+	↓
2	Nil	2.8 MID ₅₀	+	+	+	+	±	↓

^ap27 in plasma as determined by ELISA. Values above or below the cut-off value (20 pg/ml) were expressed as positive (+) or negative (–), respectively.

^bPlasma-associated virus load (RNA equivalent/ml) as determined by bDNA (cut-off: 1,500 RNA copies/ml) until week 14 post-challenge, by bDNA and QT RNA-PCR (cut-off: 50 RNA copies/ml) between week 18 and 28 post-challenge, and only by QT-RNA-PCR since week 35. Results above or below the cutoff values were expressed as positive (+) or negative (–), respectively.

^cProviral DNA copies/μg of DNA as determined by semiquantitative DNA PCR analysis. +, proviral copy number >1.

^dSHIV89.6P virus isolation from monkeys CD8-depleted PBMCs as determined by coculture with CEMx174 and measure of p27 production (cut-off = 20 pg/ml).

^ePlasma anti-SIV Ab was determined by ELISA; levels over, equal, or below the cut-off value mean of negative controls (plus 3 SD) were expressed as positive (+), border line (±), or negative (–), respectively.

^fPeripheral blood counts of CD4+ cells as evaluated by flow cytometry. The symbols ↓ and =, respectively, indicate decrease (>50% decline as compared to pre-challenge values) or normality of CD4 T-cell numbers.

* (+) positive DNA PCR was detected only sporadically and at low copy number (<10/μg of DNA).

For further technical details refer to Cafaro *et al.*, 2001.

challenge correlated in all the animals with Tat-specific CTL responses.

Thus, in both arms Tat vaccination was safe and immunogenic, and 9 out of 12 vaccinated monkeys were protected after challenge with the highly pathogenic SHIV89.6P virus (Cafaro *et al.*, 1999, 2000, 2001).

Although not inducing a sterilizing protection, Tat-based vaccines appear to contain virus replication to undetectable levels. Notably, containment almost entirely correlated with the presence of cellular immunity, in particular with anti-Tat CTL activity and Tat-induced TNF- α production also in absence of a relevant humoral response, particularly for DNA immunized animals (Cafaro *et al.*, 1999, 2001). Finally, protection was maintained for the entire follow-up (2 years), and persisted even upon boost of the animals with the recall Ag tetanus toxoid (TT), which is known to rescue virus replication (Maggiorella *et al.*, submitted).

Similar results were reported with a vaccination study in which cynomolgous monkeys immunized with viral vectors (Semliki Forest virus and modified vaccinia Ankara virus) expressing the SIV-Tat and -Rev, were protected upon challenge with a pathogenic SIV (Osterhaus *et al.*, 1999, and personal communication).

In contrast with these results, two recent studies in rhesus macaques indicate that vaccination with Tat protein (Silveira *et al.*, 2002) or tat DNA (Allen *et al.*, 2002) was safe and immunogenic, but failed to induce a significant control of viral replication following challenge with SIV or SHIV89.6P pathogenic viruses. However, several differences in the study design including dose and source of Tat (SIV and HIV), monkey species, adjuvant, dose, and schedule of immunization, dose and route of challenge viruses inoculation may account for the different results.

CONCLUDING REMARKS

Taken together, these data indicate that the immunization with Tat may control HIV replication in both vaccinated individuals exposed to the virus and seropositive patients, thus favoring the control of infection by specific immune response. Soon after entry, and prior to initiation of virus integration, the regulatory proteins Tat and Nef are expressed (Wu and Marsh, 2001), and may be recognized and killed by effector lymphocytes, induced by specific vaccination. Suppression of viral replication by CD8-mediated noncytolytic activity may also participate to an effective immune response. Thus, a limited and almost undetectable (as indicated by our monkey studies) viral replication may occur, which, in the absence of pathogenicity, may provide antigenic exposure sufficient to drive an antiviral immune response. Consequently, the anti-HIV immune response would be boosted (against Tat), broadened, and strengthened (against the other viral proteins), inducing protection to further challenges with the same or other virus strains. This hypothesis has recently been confirmed in experiments in which monkeys have been boosted with TT or rechallenged with a high virus dose (Maggiorella *et al.*, submitted, and S. Baroncelli, M.T. Maggiorella, S. Moretti, *et al.*, in preparation). In addition, two new ongoing vaccination protocols in monkeys have confirmed in a larger number of ani-

mals the data of safety and immunogenicity already obtained with the Tat protein or DNA (our unpublished data).

All these results indicate that a Tat-based vaccine is a promising candidate for vaccination in humans. Therefore, both preventive and therapeutic phase I clinical trials are currently being organized and will start in Italy within the year 2002. In addition, background (immunologic and virologic) and feasibility studies are ongoing in African countries where subsequent clinical studies (phase II and III) are being planned. Results from these studies indicate that sera from Ugandan and South African patients infected by different HIV strains recognize Tat from clade B at the same extent as individuals infected with A, B, and C clades (Buttò *et al.*, in preparation). Finally, novel approaches with Tat combined with other viral Ags (Gag, Env), delivered by the mucosal or parenteral route in prime-boost regimens, are under evaluation in preclinical studies to develop new and combined vaccine strategies in which also the Tat-adjuvant capabilities are exploited.

ACKNOWLEDGMENTS

We thank A. Comini for the veterinary assistance; E. Ialc, F. Incitti, F. Varano, N. Verrone, A. Marini, A. Avitabile, M. Chiodi, M. Azzetti, and S. Alessandrini, for hematoclinical analysis of cynomolgus samples and for the handling of the animal facility; R. Belli, F. De Angelis, M. Pace, E. Salvi, C. Rovetto, F. Carlini, M.G. Mancini, S. Fercomeni, and D. Fulgenzi (Laboratory of Virology, Istituto Superiore di Sanità) for technical help. We also thank A. Lippa and P. Sergiampietri for the editorial assistance. This work was supported by grants from the Istituto Superiore di Sanità (IX AIDS Project), the Italian Concerted Action on HIV-AIDS vaccine development (ICAV), and from the Associazione Nazionale per la Lotta contro l'AIDS (ANLAIDS), Italy.

REFERENCES

- ADD0, M.M., ALTFELD, M., ROSENBERG, E.S., ELDRIDGE, R.L., PHILIPS, M.N., HABEED, K., KHATRI, A., BRANDER, C., ROBINS, G.K., MAZZARA, G.P., GOULDER, P.J., and WALKER, B.D. (2001). The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1 infected individuals. *Proc. Natl. Acad. Sci. USA* 98, 1781-1786.
- ALLEN, T.M., MORTARA, L., MOTHE, B.R., LIEBL, M., JING, P., CALORE, B., PIEKARCZYK, M., RUDDERSDORF, R., O'CONNOR, D.H., WANG, X., WANG, C., ALLISON, D.B., ALTMAN, J.D., SETTE, A., DESROSIERS, R.C., SUTTER, G., and WATKINS, D.I. (2002). Tat-vaccinated macaques do not control simian immunodeficiency virus SIVmac239 replication. *J. Virol.* 76, 4108-4112.
- ALLEN, T.M., O'CONNOR, D.H., JING, P., DZURIS, J.L., MOTHE, B.R., VOGEL, T.U., DUNPHY, E., LIEBL, M.E., EMERSON, C., WILSON, N., KUNSTMAN, K.J., WANG, X., ALLISON, D.B., HUGHES, A.L., DESROSIERS, R.C., ALTMAN, J.D., WOLIN, S.M., SETTE, A., and WATKINS, D.I. (2000). Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* 407, 386-390.
- ARYA, S.K., GUO, C., JOSEPHS, S.F., and WONG-STAAAL, F. (1985). Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* 229, 69-73.

EFFICACY OF HIV-1 TAT-BASED VACCINATION TRIALS

609

- BABA, T.W., JEONG, Y.S., PENNICK, D., BRONSON, R., GREENE, M.P., and RUPRECHT, R.M. (1995). Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* 267, 1820-1825.
- BARILLARI, G., BUONAGURO, L., FIORELLI, V., HOFFMAN, J., MICHAELS, F., GALLO, R.C., and ENSOLI, B. (1992). Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression. Implications for AIDS-Kaposi's sarcoma pathogenesis. *J. Immunol.* 149, 3727-3734.
- BELL, D., YOUNG, J.W., and BANCHEREAU, J. (1999). Dendritic cells. *Adv. Immunol.* 72, 255-324.
- CAFARO, A., TITTI, F., FRACASSO, C., MAGGIORELLA, M.T., BARONCELLI, S., CAPUTO, A., GOLETTI, D., BORSETTI, A., PACE, M., FANALES-BELASIO, E., RIDOLFI, B., NEGRI, D.R., SERNICOLA, L., BELLI, R., CORRIAS, F., MACCHIA, I., LEONE, P., MICHELINI, Z., TEN HAAFT, P., BUTTO, S., VERANI, P., and ENSOLI, B. (2001). Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). *Vaccine* 19, 2862-2877.
- CAFARO, A., CAPUTO, A., FRACASSO, C., MAGGIORELLA, M.T., GOLETTI, D., BARONCELLI, S., PACE, M., SERNICOLA, L., KOANGA-MOGTOMO, M.L., BETTI, M., BORSETTI, A., BELLI, R., AKERBLUM, L., CORRIAS, F., BUTTO, S., HENNEY, J., VERANI, P., TITTI, F., and ENSOLI, B. (1999). Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. *Nat. Med.* 5, 643-650.
- CAFARO, A., CAPUTO, A., MAGGIORELLA, M.T., BARONCELLI, S., FRACASSO, C., PACE, M., BORSETTI, A., SERNICOLA, L., NEGRI, D.R., TEN HAAFT, P., BETTI, M., MICHELINI, Z., MACCHIA, I., FANALES-BELASIO, E., BELLI, R., CORRIAS, F., BUTTO, S., VERANI, P., TITTI, F., and ENSOLI, B. (2000). SHIV89.6P pathogenicity in cynomolgus monkeys and control of viral replication and disease onset by human immunodeficiency virus type 1 Tat vaccine. *J. Med. Primatol.* 29, 193-208.
- CALAROTA, S., BRATT, G., NORDLUND, S., HINKULA, J., LEANDERSSON, A.C., SANDSTROM, E., and WAHREN, B. (1998). Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* 351, 1320-1325.
- CALAROTA, S.A., LEANDERSSON, A.C., BRATT, G., HINKULA, J., KLINMAN, D.M., WEINHOLD, K.J., SANDSTROM, E., and WAHREN, B. (1999). Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *J. Immunol.* 163, 2330-2338.
- CAPUTO, A., BETTI, M., ALTAVILLA, G., BONACCORSI, A., BOARINI, C., MARCHISIO, M., BUTTO, S., SPARNACCI, K., LAUS, M., TONDELLI, L., and ENSOLI, B. (2002). Micellar-type complexes of tailor-made synthetic block copolymers containing the HIV-1 tat DNA for vaccine application. *Vaccine* 20, 2303-2317.
- CAPUTO, A., GAVIOLI, R., ALTAVILLA, G., BROCCA-COFANO, E., BOARINI, C., BETTI, M., CASTALDELO, A., LORENZINI, F., MICHELETTI, F., CAFARO, A., SPARNACCI, K., LAUS, M., TONDELLI, L., and ENSOLI, B. (in press). Immunization with HIV-1 tat DNA delivered by novel cationic block copolymers greatly enhances the CTL responses against Tat at very low DNA doses. *J. Immunol.*
- CASELLI, E., BETTI, M., GROSSI, M.P., BALBONI, P.G., ROSSI, C., BOARINI, C., CAFARO, A., BARBANTI-BRODANO, G., ENSOLI, B., and CAPUTO, A. (1999). DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. *J. Immunol.* 162, 5631-5638.
- CHANG, H.C., SAMANIEGO, F., NAIR, B.C., BUONAGURO, L., and ENSOLI, B. (1997). HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *AIDS* 11, 1421-1431.
- CHANG, H.K., GALLO, R.C., and ENSOLI, B. (1995). Regulation of cellular gene expression and function by the human immunodeficiency virus type 1 Tat protein. *J. Biomed. Sci.* 2, 189-202.
- CHANG, H.K., GENDELMAN, R., LISZIEWICZ, J., GALLO, R.C., and ENSOLI, B. (1994). Block of HIV-1 infection by a combination of antisense tat RNA and TAR decoys: A strategy for control of HIV-1. *Gene Ther.* 1, 208-216.
- DAVIS, D., MOREIN, B., AKERBLUM, L., LOVGREN-BENGTS-SON, K., VAN GILS, M.E., BOGERS, W.M., TEEUWSEN, V.J., and HEENBY, J.L. (1997). A recombinant prime, peptide boost vaccination strategy can focus the immune response on to more than one epitope even though these may not be immunodominant in the complex immunogen. *Vaccine* 15, 1661-1669.
- DAYTON, A.I., SODROSKI, J.G., ROSEN, C.A., GOH, W.C., and HASELTINE, W.A. (1986). The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* 44, 941-947.
- DEROSI, D., CHASSAING, G., and PROCHIANTZ, A. (1998). Trojan peptides: The penetratin system for intracellular delivery. *Trends Cell Biol.* 8, 84-87.
- DYKHUIZEN, M., MITCHEN, J.L., MONTEFIORE, D.C., THOMSON, J., ACKER, L., LARDY, H., and PAUZA, C.D. (1998). Determinants of disease in the simian immunodeficiency virus-infected rhesus macaque: Characterizing animals with low antibody responses and rapid progression. *J. Gen. Virol.* 79, 2461-2467.
- ENSOLI, B., BARILLARI, G., SALAHUDDIN, S.Z., GALLO, R.C., and WONG-STAAAL, F. (1990). Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* 345, 84-86.
- ENSOLI, B., BUONAGURO, L., BARILLARI, G., FIORELLI, V., GENDELMAN, R., MORGAN, R.A., WINGFIELD, P., and GALLO, R.C. (1993). Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol.* 67, 277-287.
- ENSOLI, B., and CAFARO, A. (2001). Novel strategies toward the development of an effective vaccine to prevent Human Immunodeficiency Virus infection or Acquired Immunodeficiency Virus. In *AIDS Clinical Review*. Volberding and Jacobson eds. pp. 23-61.
- EZZEL, C. (2002). The monkey's got AIDS: what now for live AIDS vaccine? *J. NIH Res.* 9, 21-22.
- FANALES-BELASIO, E., MORETTI, S., NAPPI, F., BARILLARI, G., MICHELETTI, F., CAFARO, A., and ENSOLI, B. (2002). Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. *J. Immunol.* 168, 197-206.
- FISHER, A.G., FEINBERG, M.B., JOSEPHS, S.F., HARPER, M.E., MARSELLE, L.M., REYES, G., GONDA, M.A., ALDOVINI, A., DEBOUK, C., and GALLO, R.C. (1986). The trans-activator gene of HTLV-III is essential for virus replication. *Nature* 320, 367-371.
- FRANKEL, A.D. and PABO, C.O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 55, 1189-1193.
- GOLDSTEIN, G., TRIBBICK, G., and MANSON, K. (2001). Two B cell epitopes of HIV-1 Tat protein have limited antigenic polymorphism in geographically diverse HIV-1 strains. *Vaccine* 19, 1738-1746.
- HINKULA, J., SVANHOLM, C., SCHWARTZ, S., LUNDHOLM, P., BRYTTING, M., ENGSTROM, G., BENTHIN, R., GLASER, H., SUTTER, G., KOHLEISEN, B., ERFLE, V., OKUDA, K., WIGZELL, H., and WAHREN, B. (1997). Recognition of prominent viral epitopes induced by immunization with human immunodeficiency virus type 1 regulatory genes. *J. Virol.* 71, 5528-5539.
- HUANG, L., BOSCH, I., HOFMANN, W., SODROSKI, J., and PARDEE, A.B. (1998). Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with

- both macrophage-tropic and T-lymphotropic HIV-1 strains. *J. Virol.* 72, 8952-8960.
- KARLSSON, G.B., HALLORAN, M., LI, J., PARK, I.W., GOMILA, R., REIMANN, K.A., AXTHELM, M.K., ILIFF, S.A., LETVIN, N.L., and SODROSKI, J. (1997). Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus monkeys. *J. Virol.* 71, 4218-4225.
- KIM, D.T., MITCHELL, D.J., BROCKSTEDT, D.G., FONG, L., NOLAN, G.P., FATHMAN, C.G., ENGLEMAN, E.G., and ROTHBARD, J.B. (1997). Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. *J. Immunol.* 159, 1666-1668.
- KLINMAN, D.M., VERTHELYI, D., TAKESHITA, F., and ISHII, K.J. (1999). Immune recognition of foreign DNA: A cure for bioterrorism? *Immunity* 11, 123-129.
- LI, C.J., UEDA, Y., SHI, B., BORODYANSKY, L., HUANG, L., LI, Y.Z., and PARDEE, A.B. (1997). Tat protein induces self-perpetuating permissivity for productive HIV-1 infection. *Proc. Natl. Acad. Sci. USA* 94, 8116-8120.
- MOY, P., DAIKH, Y., PEPINSKY, B., THOMAS, D., FAWELL, S., and BARSOUM, J. (1996). Tat-mediated protein delivery can facilitate MHC class I presentation of antigens. *Mol. Biotechnol.* 6, 105-113.
- NILSSON, C., MAKITALO, B., BERGLUND, P., BEX, F., LILJESTROM, P., SUTTER, G., ERFLE, V., TEN HAAFT, P., HEENEY, J., BIBERFELD, G., and THORSTENSSON, R. (2001). Enhanced simian immunodeficiency virus-specific immune responses in macaques induced by priming with recombinant Semliki Forest virus and boosting with modified vaccinia virus Ankara. *Vaccine* 19, 3526-3536.
- OSTERHAUS, A.D., VAN BAALEN, C.A., GRUTERS, R.A., SCHUTTEN, M., SIEBELINK, C.H., HULSKOTTE, E.G., TIJHAAR, E.J., RANDALL, R.E., VAN AMERONGEN, G., FLEUCHAUS, A., ERFLE, V., and SUTTER, G. (1999). Vaccination with Rev and Tat against AIDS. *Vaccine* 17, 2713-2714.
- PARREN, P.W., MOORE, J.P., BURTON, D.R., and SATTENTAU, Q.J. (1999). The neutralizing antibody response to HIV-1: Viral evasion and escape from humoral immunity. *AIDS* 13, S137-S162.
- PAUZA, C.D., TRIVEDI, P., WALLACE, M., RUCKWARDT, T.J., LE BUANE, H., LU, W., BIZZINI, B., BURNY, A., ZAGURY, D., and GALLO, R.C. (2000). Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. *Proc. Natl. Acad. Sci. USA* 97, 3515-3519.
- RE, M.C., FURLINI, G., VIGNOLI, M., RAMAZZOTTI, E., RODERIGO, G., DE, R., V., ZAULI, G., LOLL, S., CAPITANI, S., and LA PLACA, M. (1995). Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 10, 408-416.
- REIMANN, K.A., LI, J.T., VEAZEY, R., HALLORAN, M., PARK, I.W., KARLSSON, G.B., SODROSKI, J., and LETVIN, N.L. (1996). A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70, 6922-6928.
- REISS, P., LANGE, J.M., DE RONDE, A., DE WOLF, F., DEKKER, J., DEBOUCK, C., and GOUDSMIT, J. (1990). Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. *J. Med. Virol.* 30, 163-168.
- RODMAN, T.C., TO, S.E., HASHISH, H., and MANCHESTER, K. (1993). Epitopes for natural antibodies of human immunodeficiency virus (HIV)-negative (normal) and HIV-positive sera are coincident with two key functional sequences of HIV Tat protein. *Proc. Natl. Acad. Sci. USA* 90, 7719-7723.
- SECCHIERO, P., ZELLA, D., CAPITANI, S., GALLO, R.C., and ZAULI, G. (1999). Extracellular HIV-1 tat protein up-regulates the expression of surface CXCR4 chemokine receptor 4 in resting CD4+ T cells. *J. Immunol.* 162, 2427-2431.
- SILVERA, P., RICHARDSON, M.W., GREENHOUSE, J., YALLEY-OGUNRO, J., SHAW, N., MIRCHANDANI, J., KHALIL, K., ZAGURY, J.F., LEWIS, M.G., and RAPPAPORT, J. (2002). Outcome of simian-human immunodeficiency virus strain 89.6p challenge following vaccination of rhesus macaques with human immunodeficiency virus Tat protein. *J. Virol.* 76, 3800-3809.
- TYAGI, M., RUSNATI, M., PRESTA, M., and GIACCA, M. (2001). Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 276, 3254-3261.
- VAN BAALEN, C.A., PONTESILLI, O., HUISMAN, R.C., GERETTI, A.M., KLEIN, M.R., DE WOLF, F., MIEDEMA, F., GRUTERS, R.A., and OSTERHAUS, A.D. (1997). Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. *J. Gen. Virol.* 78, 1913-1918.
- WHATMORE, A.M., COOK, N., HALL, G.A., SHARPE, S., RUD, E.W., and CRANAGE, M.P. (1995). Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence. *J. Virol.* 69, 5117-5123.
- WU, Y., and MARSH, J.W. (2001). Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science* 293, 1503-1506.
- ZAGURY, J.F., SILL, A., BLATTNER, W., LACHGAR, A., LE BUANE, H., RICHARDSON, M., RAPPAPORT, J., HENDEL, H., BIZZINI, G., GRINGERI, A., CARCAGNO, M., CRISCUOLO, M., BURNY, A., GALLO, R.C., and ZAGURY, D. (1998). Antibodies to the HIV-1 Tat protein correlated with nonprogression to AIDS: A rationale for the use of Tat toxoid as an HIV-1 vaccine. *J. Hum. Virol.* 1, 282-292.

Address reprint requests to:
Barbara Ensoli, M.D., Ph.D.
Retrovirus Division
Laboratory of Virology
Istituto Superiore di Sanità
Viale Regina Elena 299
00161 Rome, Italy

E-mail: ensoli@iss.it

88328963. PubMed ID: 2970960. Cellular processing of pro-atrial natriuretic factor (pro-ANF): studies using an antiserum that selectively binds ANF-(99-126) after its cleavage from pro-ANF. **Wildeck G M**; Fischman A J; Fallon J T; Matsueda G R; Zisfein J B; Preibisch G; Seipke G; Homcy C J; Graham R M. (Cellular and Molecular Research Laboratory, Massachusetts General Hospital, Boston 02114.) Endocrinology, (1988 Oct) Vol. 123, No. 4, pp. 2054-61. Journal code: 0375040. ISSN: 0013-7227. Pub. country: United States. Language: English.

AB Atrial natriuretic factor (ANF) is stored in atrial myocytes as a 15-17K prohormone, but circulates in plasma as a 3K, carboxy (C)-terminal fragment of the prohormone. The tissue location at which the cleavage of pro-ANF to its hormonal form occurs is unknown. In the present study, an immunological approach was taken to address this question. A polyclonal antiserum was generated which recognizes the hormonal form of ANF [ANF-(99-126)] only after its cleavage from the prohormone. This was accomplished by immunizing rabbits with a synthetic peptide corresponding to the seven amino (N)-terminal residues of ANF-(99-126) coupled to carrier protein via a C-terminal cysteine. This antiserum, anti-ANF-(99-105), demonstrated high affinity for ANF-(99-126) (IC50 = 170 pM), but displayed 100-fold less affinity for recombinant pro-ANF [ANF-(2-126)]. The N-terminal specificity of anti-ANF-(99-105) was evident by its failure to bind ANF-(103-126) at concentrations up to 100 nM. The specificity of anti-ANF-(99-105) for the hormonal form of ANF was examined by using thrombin to cleave pro-ANF and testing for the generation of anti-ANF-(99-105) immunoreactivity. Cleavage of atrial pro-ANF or 35S biosynthetically-labeled pro-ANF resulted in the production of immunoreactive material from the prohormone, whereas pro-ANF itself demonstrated no cross-reactivity with anti-ANF-(99-105). Anti-ANF-(99-105) could also recognize ANF released from the isolated perfused rat heart. When anti-ANF-(99-105) was used in immunohistochemical studies of rat atrial myocardium, no staining was observed in unfixed frozen sections. This suggests that proteolytic processing of pro-ANF is not an intracardiocytic event.

M

Acetylation of HIV-1 Tat by CBP/p300 Increases Transcription of Integrated HIV-1 Genome and Enhances Binding to Core Histones

Longwen Deng,* Cynthia de la Fuente,* Peng Fu,* Lai Wang,* Robert Donnelly,† John D. Wade,‡ Paul Lambert,§ Hong Li,* Chee-Gun Lee,* and Fatah Kashanchi*¹

*Department of Biochemistry and Molecular Biology, and †Department of Pathology, UMDNJ–New Jersey Medical School, MSB E-635, Newark, New Jersey 07103; ‡Howard Florey Institute, University of Melbourne, Parkville, Victoria 3052, Australia; and §School of Medicine, Flinders University of South Australia, Bedford Park, South Australia 5042, Australia

Received April 7, 2000; returned to author for revision June 28, 2000; accepted August 4, 2000

The HIV-1 Tat protein is required for viral replication and is a potent stimulator of viral transcription. Although Tat has been extensively studied in various reductive paradigms, to date there is little information as to how this activator mediates transcription from natural nucleosomally packaged long terminal repeats. Here we show that CREB-binding protein (CBP)/p300 interacts with the HIV-1 Tat protein and serves as a coactivator of Tat-dependent HIV-1 gene expression on an integrated HIV-1 provirus. The site of acetylation of Tat was mapped to the double-lysine motif in a highly conserved region, "RKKRRQ", of the basic RNA-binding motif of Tat. Using HLM1 cells (HIV-1⁺/Tat⁺), which contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, we find that only wild type, and not K50A, K51A, or K50A/K51A alone or in combination of ectopic CBP/p300, is able to produce full-length infectious virions, as measured by p24 gag ELISAs. Tat binds CBP/p300 in the minimal histone acetyltransferase domain (1253–1710) and the binding is stable up to 0.85 M salt wash conditions. Interestingly, wild-type peptide 41–54, and not other Tat peptides, changes the conformation of the CBP/p300 such that it can acquire and bind better to basal factors such as TBP and TFIIB, indicating that Tat may influence the transcription machinery by helping CBP/p300 to recruit new partners into the transcription machinery. Finally, using biotinylated wild-type or acetylated peptides, we find that acetylation decreases Tat's ability to bind the TAR RNA element, as well as to bind basal factors such as TBP, CBP, Core-Pol II, or cyclin T. However, the acetylated Tat peptide is able to bind to core histones on a nucleosome assembled HIV-1 proviral DNA. © 2000 Academic Press

INTRODUCTION

The HIV-1 Tat protein is required for viral replication and is a potent stimulator of viral transcription. Tat stimulates viral gene expression through an RNA element in the viral long terminal repeat (LTR). For optimal transactivation of HIV-1 gene expression, Tat requires specific upstream transcription factors, including Sp1 (Jeang *et al.*, 1993), TATA-binding protein (Kashanchi *et al.*, 1994b; Veschambre *et al.*, 1995; Majello *et al.*, 1998), Tat-associated kinase (Herrmann and Rice, 1995; Yang *et al.*, 1996), TFIIB (Garcia-Martinez, *et al.*, 1997; Parada and Roeder, 1996), Tip (Jeang *et al.*, 1993; Henderson *et al.*, 1999), and RNA polymerase II (Cujec *et al.*, 1997; Mavankal *et al.*, 1996). The ability of Tat to regulate viral transcription is related to its ability to interact with the basal transcription complexes responsible for the initiation of transcription including cyclinT/cdk9 complex, resulting in a more efficient elongating RNA Pol II complex (O'Keefe *et al.*, 2000; Romano *et al.*, 1999; Napolitano *et al.*, 1999; Isel and Karn, 1999; Bieniasz *et al.*, 2000; Ramathanan *et al.*, 1999; Ivanov *et al.*, 1999; Chen *et al.*,

1999a; Wimmer *et al.*, 1999; Garriga *et al.*, 1998; Garber *et al.*, 1998; Fujinaga *et al.*, 1998; Wei *et al.*, 1998).

Among the factors associated with basal transcription complexes, CBP (CREB-binding protein) and p300 have emerged as coactivators for various DNA-binding transcription factors. CBP and p300 are large proteins, 2441 and 2414 amino acids, respectively, that have the ability to interact simultaneously with various transcription factors such as nuclear hormone receptors, CREB, c-Jun, v-Jun, c-Myb, v-Myb, Sap-1a, c-Fos, MyoD, YY1, NF- κ B, and p53 (Goldman *et al.*, 1997) and with other coactivators such as P/CAF (Blanco *et al.*, 1998; Chakravarti *et al.*, 1999), as well as with basal components of the transcriptional apparatus. Therefore, it is this wide array of functions that have allowed CBP/p300 proteins to be important transcriptional integrators (Shikama *et al.*, 1999). In recent years multiple mechanisms have emerged for the function of CBP/p300. The first mechanism for CBP/p300 activation involves the acetylation of the terminal tails of the core histones by histone acetyltransferase (HAT) and destabilization of histone–DNA interactions, allowing transcription factors access to the promoter region. The second mechanism by which CBP has been suggested to be an activator of transcription is by bridging the gap between upstream DNA-bound transcription factors and

¹ To whom correspondence and reprint requests should be addressed. E-mail: Kashanchi@umdnj.edu.

components of the general transcription machinery. The third possible mechanism is CBP/p300's ability to directly acetylate nonhistone proteins such as p53 (Gu and Roeder, 1997), the erythroid Kruppel-like factor (EKLF, Zhang and Bieker, 1998), the nuclear hormone receptor coactivators ACTR (Chen *et al.*, 1997), and the basal transcription factor TFIIE and TFIIF (Imhof *et al.*, 1997; Martinez-Balbas *et al.*, 1998). In the case of p53, acetylation of the regulatory domain led to a dramatic increase in DNA binding *in vitro*, whereas the acetylation of ACTR by CBP/p300 disrupts the receptor-coactivator interaction, which plays a key role in hormone-induced gene activation (Chen *et al.*, 1999b).

HIV-1 proviral DNA is integrated into host cell chromosomes and packaged into chromatin. The LTR acts as a very strong promoter when analyzed as naked DNA *in vitro* and is silent when integrated into the cellular host genome in the absence of any stimuli (Verdin, 1991; Adams *et al.*, 1994; Van Lint *et al.*, 1996; Marzio *et al.*, 1998; Benkirane *et al.*, 1998). Recently, several reports have shown the existence of an intracellular multiprotein complex that contains Tat, CBP/p300, and P/CAF. It was found that the histone acetyltransferase activity of CBP/p300 and P/CAF is preferentially required for Tat function (Kiernan *et al.*, 1999). CBP/p300 was also recently reported to interact with the HIV-1 Tat protein and serves as a coactivator of Tat-dependent HIV-1 gene expression (Hottiger *et al.*, 1998; Ott *et al.*, 1999). This superinduction has been attributed to the histone acetyltransferase (HAT) activity of CBP/p300 on the integrated HIV-1 promoter.

In this study, we find that Tat is acetylated by CBP/p300 and mapped to the double-lysine motif in a highly conserved region (⁴⁹RKKRRQ⁵⁴) of the Tat protein. Using HLM1 (HIV-1⁺/Tat⁻) cells, which contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, we find that only wild type, and not K50A, K51A, or a double-mutant K50A/K51A alone or in combination with excess CBP/p300, is able to produce full-length infectious virions. Furthermore, mechanistically, the wild-type Tat peptide 41–54, which contains the basic core domain of HIV-1 Tat, changes the conformation of CBP/p300 such that basal factors such as TBP and TFIIB bind better to CBP/p300, indicating that Tat may influence the transcription machinery by helping CBP/p300 to acquire new partners in the transcription machinery.

Tat binds to CBP/p300 minimal HAT domain (1253–1710) and is a stable complex up to 0.85 M salt wash conditions. Acetylation of Tat by CBP/p300 decreases Tat's ability to bind the TAR RNA element *in vitro*. Finally, using biotinylated wild-type or acetylated Tat peptides, we find that acetylation causes a release of Tat from basal factors such as TBP, CBP, or cyclin T. Interestingly, the acetylated Tat peptide is able to bind with higher affinity to core histones on nucleosomal DNA.

RESULTS

HIV-1 Tat is acetylated by the HAT domain of CBP/p300

We initially asked whether a minimal HAT domain of CBP/p300 was capable of acetylating the Tat protein *in vitro*. To answer this question, we constructed a minimal GST–HAT plasmid from a full-length p300 cDNA clone, expressed in *Escherichia coli* and purified using glutathione-agarose beads. When using GST–HAT in a [¹⁴C]acetyl coenzyme A exchange reaction, we found that GST–HAT was capable of efficient acetylation of core histones H2A, H2B, H3, and H4 *in vitro* (Fig. 1A, lane 3). We also observed efficient acetylation of purified Tat protein *in vitro*, as shown in Fig. 1A, lane 4. Products shown in Fig. 1A are ¹⁴C-acetylated polypeptides that had been separated on 4–20% SDS–PAGE, dried, and exposed to a Phosphorimager cassette. The bottom panel of Fig. 1A shows the Coomassie blue staining of the same gel. It is important to note that we have consistently observed a more efficient acetylation of Tat proteins that do not contain a GST moiety at their N-terminus. Similar efficient *in vitro* acetylations were also observed with histidine- and epitope (influenza)-tagged peptides at the N- or C-terminus of the Tat protein (data not shown).

We next examined the effect of various Tat peptides as substrates in the *in vitro* HAT assay. The results of such an experiment are shown in Fig. 1C, where Tat 41–54 peptide, but not 41–50 peptide (lanes 7–10), was acetylated with GST–HAT. Similar to Fig. 1A, results shown in Fig. 1C are products separated on 4–20% SDS–PAGE, dried, and exposed to a cassette. It is important to note that we have observed reproducible results only when using [¹⁴C]acetyl CoA and SDS–PAGE (4–20%) for separation purposes as opposed to [³H]acetyl CoA and filter disks for detection of acetylated small peptides. Peptides of such short lengths do not reproducibly bind to DE52 filter papers and cannot stand rigorous wash conditions. A summary of all the Tat peptides used in the HAT assay is shown in Fig. 1D. It is important to note that peptides such as 65–86, which contains lysine residues, were not acetylated, indicating that the *in vitro* acetylation by the CBP/p300 HAT domain is not a nonspecific reaction. Finally, we used wild-type full-length human HeLa p300 (hp300, a generous gift from R. Shiekhattar), or epitope-tagged recombinant p300 (rp300), and observed efficient acetylation of the Tat protein *in vitro* (Fig. 1E).

Site of Tat acetylation by the HAT domain

The Tat peptide 41–54 contains three lysine residues, one at position 41 and the other two at positions 50 and 51. To determine which lysine residues in the 41–54 peptide were acetylated by GST–HAT, we initially made acetylated 41–54 peptides with acetyl groups at posi-

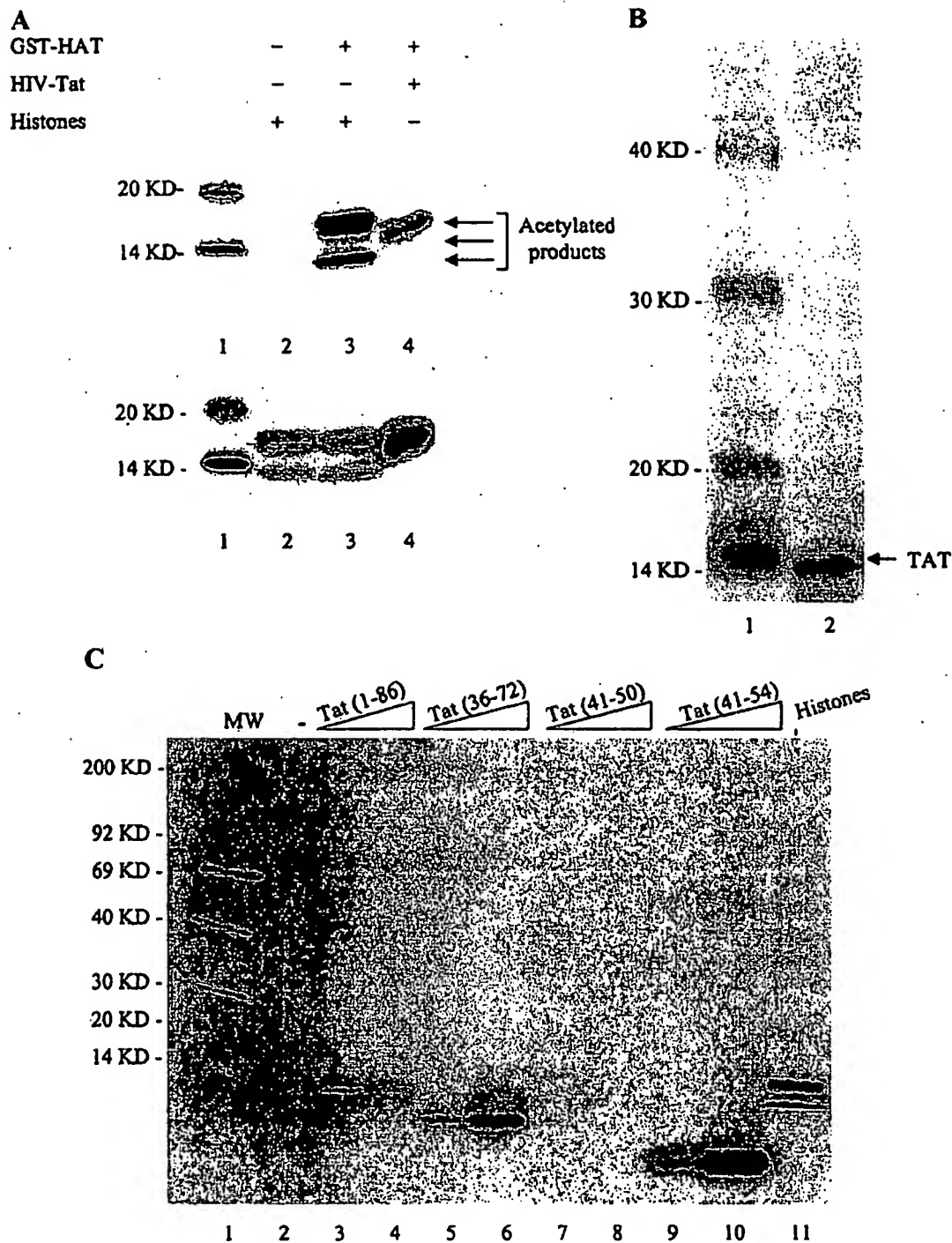
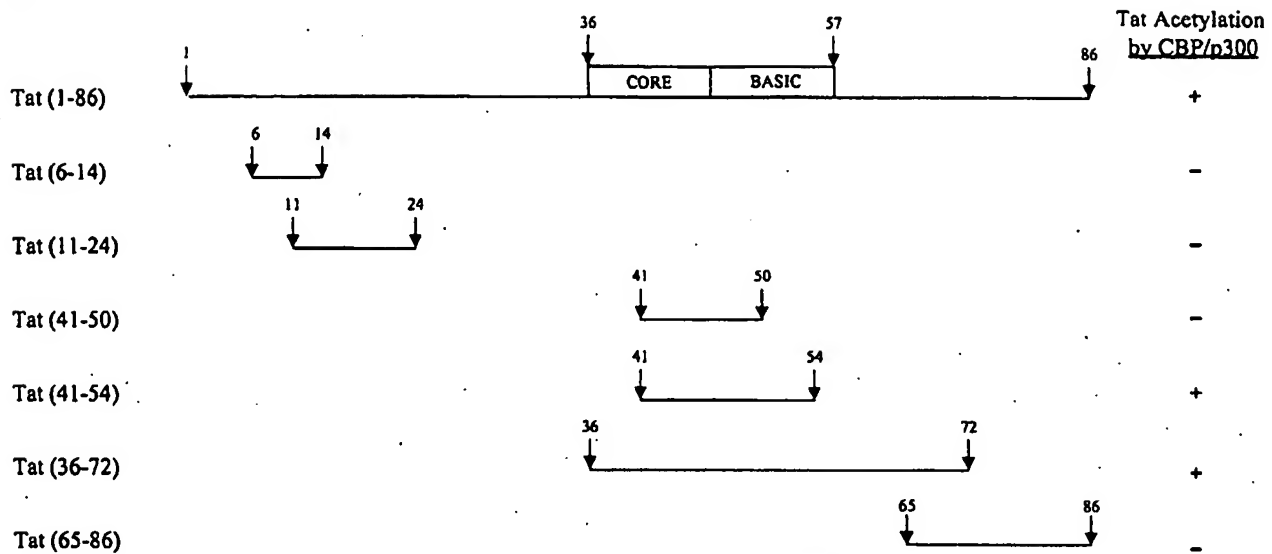


FIG. 1. Acetylation of HIV-1 Tat protein by CBP/p300 *in vitro*. (A, B) The core histones H2A, H2B, H3, and H4 (lanes 2 and 3) and Tat protein (1-86, lane 4) were incubated with or without GST-p300 (HAT domain) and [14 C]acetyl-CoA. (A) Acetylated products (lanes 3 and 4) resolved on 4-20% SDS-PAGE, dried, and exposed to a PhosphorImager cassette. (A, bottom) Coomassie blue staining of the gel shown at top. B represents the purified full-length Tat protein (1-86, 1 μ g) used in the HAT assay, resolved on a SDS-PAGE gel, and silver stained (31). (C) Tat acetylation site located at the basic RNA-binding domain. Synthesized Tat peptides covering various regions of the Tat protein (lane 3-11) were incubated with GST-p300 and [14 C]acetyl CoA and analyzed on 4-20% SDS-PAGE. Lane 2 serves as negative control with no substrate added to the reaction, and lanes 3, 4, and 11 serve as positive controls, where full-length Tat and core histone proteins were added to the reaction. All other reactions were performed with two concentrations (200 and 400 ng) of various peptides. (D) The schematic representation of Tat protein and Tat peptides used in this study and the results of acetylation by GST-p300. (E) Acetylation of HIV-1 TAT by wild-type p300/CBP. (Left) Acetylation of TAT or all four histones with 10 ng of HeLa purified CBP/p300 complexes (hp300). Proteins, BSA, wild-type TAT, or histones were incubated with HeLa CBP/p300, followed by incubation at 37°C for 1 h, spotted on DE52 filter discs, washed, and counted. (Right) A similar experiment with either TAT or free histones incubated with 10 ng of purified epitope-tagged recombinant Baculovirus p300 (rp300). Samples were incubated at 37°C for 1 h and subsequently run on 4-20% SDS-PAGE, dried, and exposed to a PhosphorImager cassette.

D



E

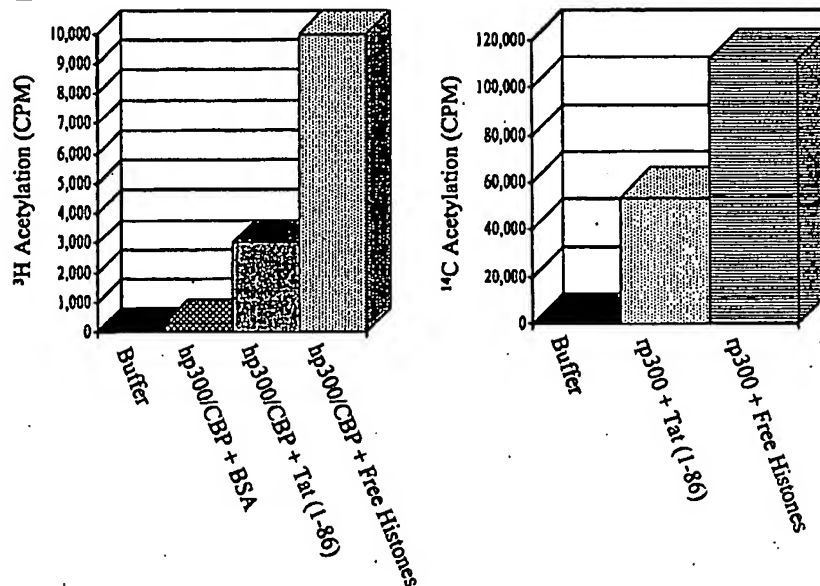


FIG. 1—Continued

tions 50, 51, or 50+51. The acetyl groups were put on the peptides during the chemical peptide synthesis. All peptides were subsequently purified on C18 reverse-phase HPLC and dried, and quantitations were determined by protein assay as well as by running small aliquots on 4–20% SDS–PAGE followed by silver stain detection. As can be seen in Fig. 2A, all peptides were efficiently acetylated with GST–HAT, except the double-acetylated 41–54 (lanes 9 and 10). The doubly acetylated 41–54 peptide was, however, very weakly acetylated at lysine 41 position, at a 10-fold excess of GST–HAT concentrations *in vitro*, and similar results were also obtained with a longer peptide, 36–58 (data not shown). Therefore, it may be possible that all three lysines are acetylated, although at very different kinetic rates, depending on the

enzyme concentrations used in these assays. To determine which lysine, 50 or 51, was acetylated efficiently *in vitro*, we synthesized a second batch of acetylated peptides starting from position 42–54. The results of such an experiment are shown in Fig. 2B, where peptide 42–54, which had already been acetylated at position 50, was a poor substrate for accepting the new acetyl group at position 51 (lanes 3 and 4). However, the peptide that was acetylated at position 51 could serve as an excellent substrate for acetylation *in vitro*. The doubly acetylated 42–54 peptide could not be acetylated at any concentration of GST–HAT (data not shown). We next examined the effect of Tat acetylation on cells expressing Tat. HeLa/eTat and HeLa/pcep4 lines contain either an epitope-tagged Tat at the C-terminus (HeLa/eTat) or a control

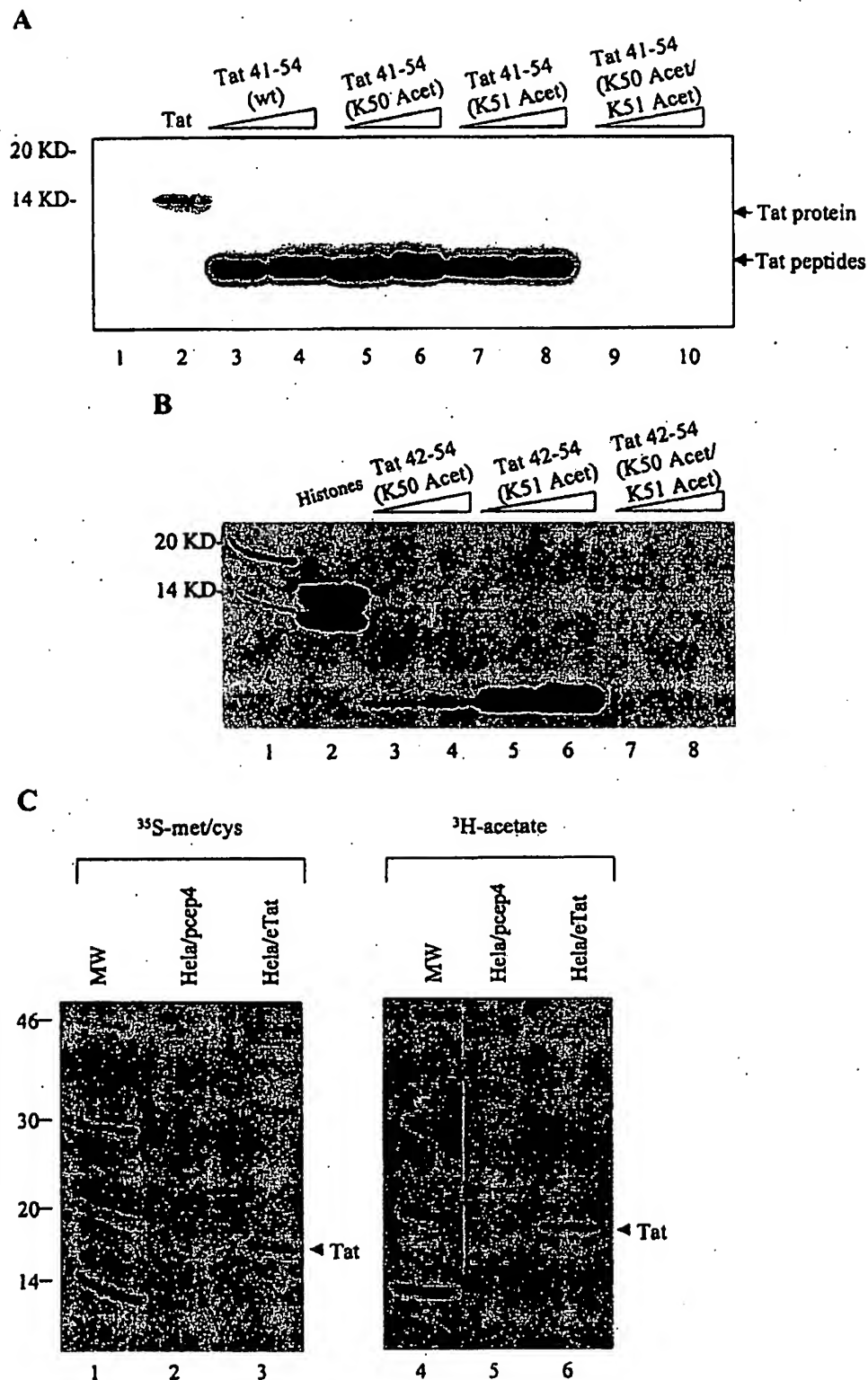


FIG. 2. Tat acetylation sites mapped to double-lysine motifs K50 and K51 in the basic RNA-binding domain. (A, B) Two concentrations (200 and 400 ng) of the Tat peptides (41–54 or 42–54), wild-type or acetylated at lysines position 50, 51, and 50+51, were incubated with GST-p300 and [¹⁴C]acetyl CoA, separated on 4–20% SDS-PAGE, and exposed to a PhosphorImager cassette. (C) Both log-phase HeLa/eTat or HeLa/pcep4 cells were labeled with [³H]acetate in DMEM complete medium plus hygromycin (left) or incubated overnight with [³⁵S]methionine/cysteine (right). Nuclear lysates were used for immunoprecipitations on cross-linked monoclonal 12CA5 Ab beads and eluted with a 100-fold excess of influenza peptide. The [³⁵S]methionine/cysteine gel was exposed overnight and the [³H]acetate gel was exposed for 1 week on the PhosphorImager cassette.

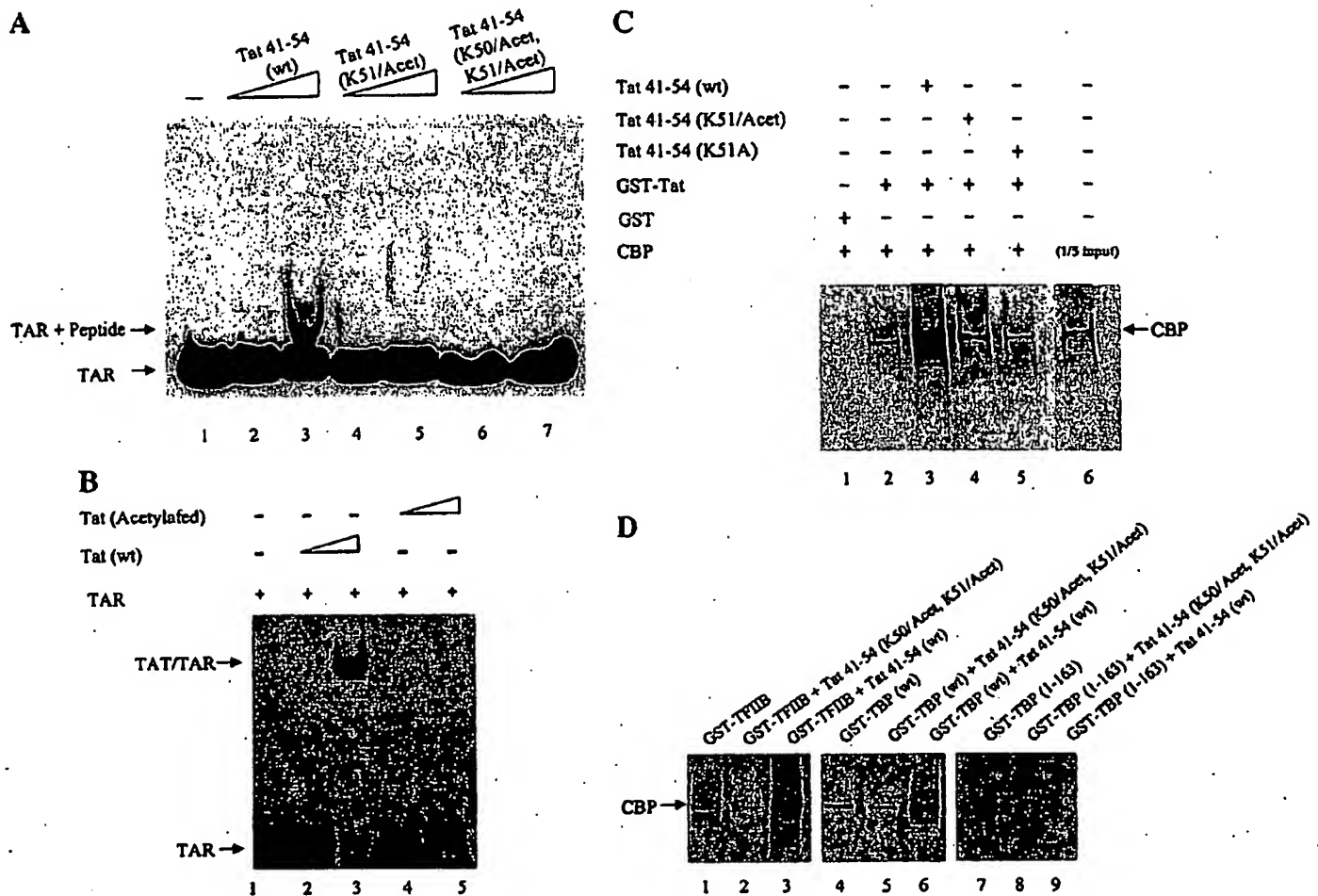


FIG. 3. Acetylated Tat decreases its ability to HIV-1 TAR RNA. (A) The wild-type Tat peptide 41–54 (lanes 2 and 3) and the acetylated Tat peptide 41–54 (lanes 4–7) were incubated with 32 P-labeled TAR RNA at room temperature for 30 min and separated on a 6% DNA retardation polyacrylamide gel (Novex). Lane 1 contains TAR RNA alone and lanes 2–7 indicate TAR plus various wild-type or acetylated peptides (2.5 and 5 μ g). (B) Wild-type (lanes 2 and 3, 0.5 and 1.0 μ g, respectively) or GST-HAT *in vitro* acetylated Tat proteins (1–86, lanes 4 and 5, 0.5 and 1.0 μ g, respectively) were incubated with TAR RNA, resolved on a 6% DNA retardation gel, dried, and exposed to a PhosphorImager cassette. (C, D) Tat peptide (41–54) increases the binding of CBP to TBP, TFIIIB, and Tat. Tat peptides, wild type or various modified versions (10 μ g), were preincubated with 35 S-labeled CBP (Promega TNT, 10 μ l) for 10 min and subsequently used to bind to 500 ng eluted GST-Tat, GST-TFIIIB, GST-TBP (wt), or GST-TBP (1–163, deletion from 164 to 337 of human TBP). After several washes (3 \times), the bound proteins were resolved by 4–20% SDS-PAGE, dried, and exposed.

backbone vector (HeLa/pcep4). Both cell types have been described previously (Kashanchi *et al.*, 2000). Log-phase-growing cells were labeled with [3 H]acetate or [35 S]methionine/cysteine, and nuclear extracts were obtained for immunoprecipitations on cross-linked 12CA5 antibody beads. Following binding, Tat was eluted with an excess of competitor peptide and run on a 4–20% gel. The results of such an experiment are shown in Fig. 2C, where acetylated Tat (right column) could be obtained only from HeLa/eTat cells and not from the control pcep4 cells.

Effect of Tat acetylation on TAR and CBP/P300 binding

We next asked whether acetylation of Tat could increase or decrease its affinity for TAR RNA. To address this question, we synthesized labeled TAR RNA, PAGE

purified it, and used it in an RNA bandshift assay. The results of such an experiment are shown in Fig. 3A, where wild-type peptide 41–54 was capable of binding to TAR RNA (lane 3). The TAR RNA binding is completely abolished when lysines 50 and 51 are acetylated (lanes 6 and 7). We observed no binding of double-acetylated 50 and 51 peptide to TAR RNA at any peptide concentration (data not shown). Similar results were also observed when Tat protein was acetylated with GST-HAT prior to TAR RNA binding (Fig. 3B, lanes 4 and 5).

We next examined the effect of wild-type or acetylated peptides on CBP/p300 binding *in vitro*. We reasoned that if only wild-type Tat peptide or protein was able to bind to TAR RNA and not the acetylated counterparts, it would then be possible for acetylated Tat to also bind less efficiently to CBP/p300. The results of such an experiment are shown in Fig. 3C, where GST-Tat, but not GST,

was able to bind to ^{35}S -labeled CBP *in vitro*. However, when performing competition experiments with wild-type, acetylated, or alanine-substituted Tat peptides in the same reaction, we found a surprising result, where wild-type 41–54 but not other derivatives was able to enhance the binding of CBP to GST-Tat (compare lanes 3–5). We have found similar results when using immunoaffinity-purified recombinant p300 from Baculovirus-infected cells (data not shown). This unexpected result suggested to us that perhaps the wild-type peptide might change the conformation of CBP/p300 such that it can bind better to other proteins. This interpretation is very likely, since Tat has been shown to dimerize and contact multiple transcription factors on the transcription initiation site. To test that hypothesis, we performed a similar experiment with CBP pretreated with either wild-type or double-acetylated peptide prior to binding to other basal factors such as TFIIB and TBP. Both TFIIB and TBP have previously been shown to bind to CBP/p300 molecules (Sang *et al.*, 1997). The results in Fig. 3D indicate that when CBP is pretreated with only wild-type Tat peptide, it can bind more efficiently to GST-TFIIB or GST-TBP, but not to a GST-TBP (1–163) mutant. The reaction was specific for a possible change of CBP conformation, since pretreatment of GST-TFIIB, TBP, or the mutant TBP with any of the peptides did not increase CBP binding *in vitro* (data not shown). Taken together, the results from TAR binding as well as CBP binding indicate that wild-type and not acetylated Tat binds to the basal transcription machinery and that acetylated Tat might either completely come off the transcriptional complex or simply stay with the elongating RNA Pol II.

Localization of CBP/p300 binding to Tat

It has previously been shown that p300 binds to Tat at core and basic residues (Marzio *et al.*, 1998; Benkirane *et al.*, 1998; Hottiger and Nabel, 1998) and that Tat binds to the HAT, N-terminal, or C-terminal domains of p300. We decided to determine which region of p300 was stably binding to the Tat protein. Initially, we used a series of four GST-CBP mutants (a generous gift from R. Goodman) and three GST-p300 mutants in an *in vitro* TNT-binding assay. Figure 4A is a general diagram that depicts the relationship between CBP and p300 molecules as well as various mutants used in our assays. When binding ^{35}S -labeled Tat to various GST-p300 domains, we found that Tat bound stably to all A, B, and C mutant domains under 0.1 M salt wash conditions (Fig. 4B, lanes 1–4). However, the binding of Tat to p300 was most resistant with the B fragment (aa 744–1540) of GST-p300. Tat retained its binding to this fragment under 0.85 M salt wash conditions (compare lanes 12–14). Similar results were obtained with the GST-CBP HAT domain (data not shown). Therefore, Tat may interact with multiple CBP/p300 domains; however, its ability to tightly associate

with the HAT domain may indicate similarity in functions with other viral HAT-binding proteins such as adenovirus E1A or SV40 large T antigen.

Functional activity of mutant Tat proteins on transient transfections

Tat is one of the most powerful viral activators known to date. Tat could stimulate transcription of HIV-1 promoter anywhere from 100- to 1000-fold depending on the assay conditions used. To determine which Tat residues (position 50, 51, or 50+51) were important for HIV-1 promoter activity, we synthesized alanine-substituted vectors and sequenced all three forms of CMV-driven Tat vectors. We subsequently used the Tat mutants in transient transfection assays using CEM (12D7) T-cells. The results of such an experiment are shown in Fig. 5A, where upon electroporation of K50A or K51A with reporter HIV-LTR CAT, we observed a slight drop in transcriptional activity (less than 50%, compare lanes 3–6). However, a more pronounced drop in transactivation was observed with the double-mutant 50 and 51 (~5-fold, lanes 7 and 8). This drop in activation from the 50+51 mutant could not be rescued with ectopic expression of CBP vector (Fig. 5B, lane 4). Similar results were obtained with a CMV-p300 expression vector (data not shown). Finally to conserve the positive charge of the lysine residues, we synthesized Tat protein with arginines at positions 50 and 51 and used the protein to perform CAT assays in CEM cells. The results in Fig. 5C indicate that only lysine residues and not alanine or arginine substitutions at positions 50 and 51 are critical for the observed acetylation effect. Taken together, the transient transfection results indicate that neither K50 nor K51 mutations alone are sufficient to completely lose the Tat transactivation on HIV-1 LTR.

Activation of integrated HIV-1 provirus requires the HAT domain of CBP/P300

We used HLM-1 cells to address whether the interaction of Tat and CBP/p300 plays a role in the activation of integrated proviral HIV-1 sequence *in vivo*. HLM-1 cells (AIDS Research and Reference Reagent Program) were derived from HeLa-T4⁺ cells containing an integrated copy of the HIV-1 proviral genome with the Tat-defective mutation (termination linker at the first AUG). HLM-1 cells are negative for virus particle production, but can be induced to express high levels of infectious HIV-1 after transfection with Tat-expressing clones or after stimulation with cytokines such as TNF- α or general inducers such as sodium butyrate. The new resulting particles are wild type for infection, RT activity, and integration, but need to be restimulated for the next round of progeny formation. In order to test whether coactivator CBP/p300 plays an important role in the activation of integrated HIV-1, we transfected the HLM-1 cells with Tat and CBP

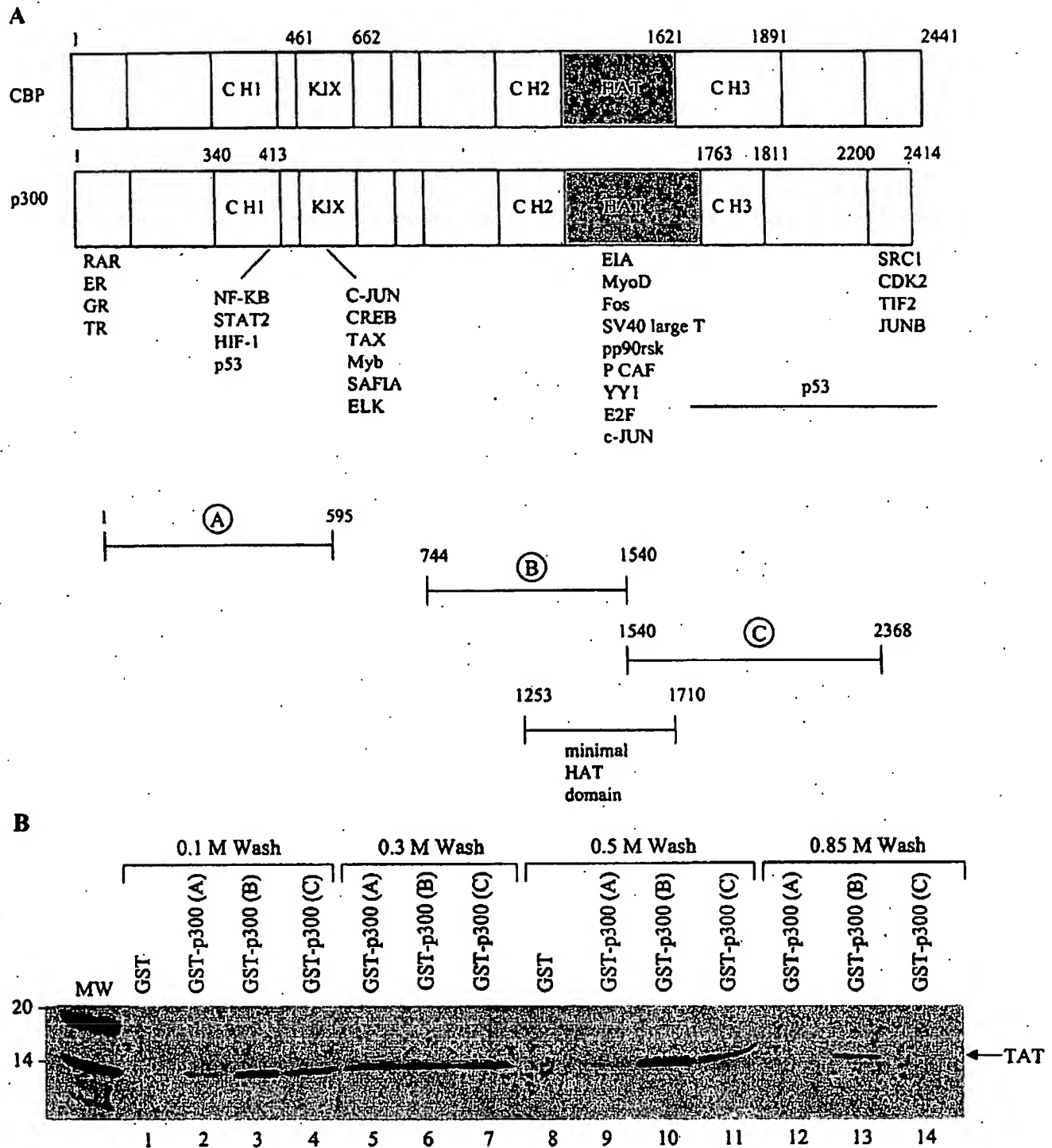
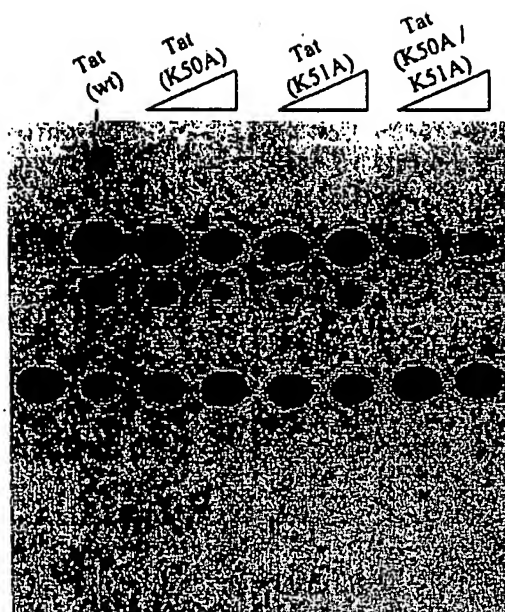


FIG. 4. Binding of Tat to CBP and p300 under various salt conditions. (A) Schematic representation of functional domains in CBP, p300, and the GST-p300 clones containing various domains used here, including GST-p300 A (1-595), GST-p300 B (744-1540), and GST-p300 C (1540-2368). (B) Binding of Tat to p300 under various salt wash conditions. The GST-p300 deletion clones (A, B, and C) were immobilized on glutathione beads from bacterial extracts and incubated with radiolabeled, *in vitro* translated ^{35}S -labeled Tat. The bound proteins were resolved on 4-20% SDS-PAGE after being washed with buffers containing TNE + 0.1, 0.3, 0.5, or 0.85 M salt and 0.1% NP-40. Tat binds to GST-p300 B and C fragments at 0.3 M salt buffer. Tat binds only to GST-p300 B fragment after a 0.85 M salt wash buffer.

and with or without E1A, which has been shown to inhibit the HAT activity of CBP/p300 (Chakravarti *et al.*, 1999). The resulting supernatants were collected at various time points and tested for the production of HIV-1 parti-

cles by p24 gag antigen ELISA. The results of such an experiment are shown in Fig. 6A, where ectopic expression of CBP along with Tat activates the viral production by four- to fivefold. Furthermore, the effect of CBP acti-

A

Lane:	1	2	3	4	5	6	7	8
% Conversion	1.5	85.0	49.9	43.8	39.1	55.2	14.4	16.6

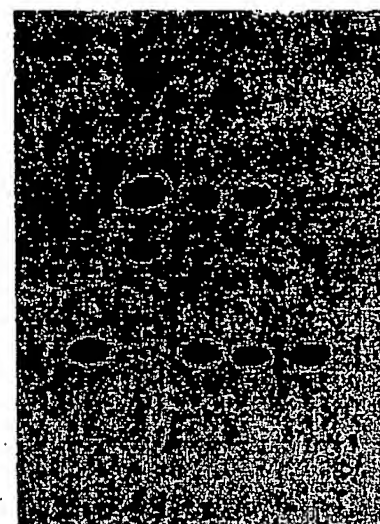
B CBP

Tat (K50A/K51A)

Tat (wt)

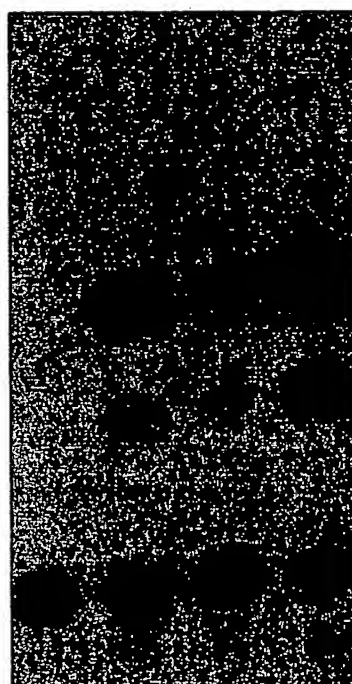
HIV LTR-CAT

-	-	-	+	+
-	-	+	+	-
-	+	-	-	-
+	+	+	+	+



Lane:	1	2	3	4	5
% Conversion	1.2	98.8	25.2	23.4	1.8

C TAT (wt)	-	-	-	+
TAT (K50/51R)	-	-	+	-
TAT (K50/51A)	-	+	-	-
HIV LTR-CAT	+	+	+	+



	1	2	3	4
% Conversion	0.9%	17%	9.2%	88%

vation can be reversed using the E1A expression vector. Interestingly, neither E1A nor CBP by itself can activate HIV-1 production, indicating at least in the case of CBP, that CBP exerts its activation effect on the HIV-1 promoter only in the context of chromatin DNA. Therefore, unlike other retroviral activation pathways, such as HTLV-1 (Kashanchi *et al.*, 1998), the HAT domain of CBP/p300 may be a crucial domain in HIV-1 proviral transcriptional activation.

We next examined the effect of the Tat mutants at positions 50 and 51 and a combination 50+51 mutant in HLM1 cells. Results presented in Fig. 6B indicate that point mutations at position 50 or 51 are equally deleterious in activation of the integrated chromatinized HIV-1 DNA. This is in marked contrast to the transfection data (Fig. 5A) where point mutants at 50 or 51 were slightly affected and only the double-mutant 50+51 vector was dramatically reduced in activity. More importantly, ectopic expression of the CBP could not activate the 50 or 51 mutants in these cells. Similar levels of nuclear Tat wild type and mutants were observed in these cells (data not shown). Finally, we performed a titration assay of the Tat 50+51 mutant with CBP and found no induction of the latent virus in these cells (Fig. 6C). Similar results were also obtained with the Tat point mutants 50 or 51 and titration of various concentrations of ectopically expressed CBP (data not shown). Taken together these data indicate that the effect of CBP/p300 is at the level of integrated HIV-1 provirus and that both lysines at position 50 and 51 are equally important for this activation pathway.

Binding target of Tat acetylated peptide

To date there are no clear examples of what exactly happens to a protein once it is acetylated in the transcriptional complex by coactivators such as CBP/p300. In fact, the majority of the existing reports on acetylated proteins discuss what the functional consequence of acetylation may be (Gu and Roeder, 1997) and not so much regarding the proteins or pathways downstream of acetylation. We decided to address this question by utilizing affinity pull-down experiments using wild-type or acetylated 42–54 peptides coupled to a biotin moiety. The biotinylated peptides were incubated with various

³⁵S-labeled proteins or nuclear extracts followed by direct radioactive detection or Western blot for transcription factors involved in HIV-1 transcription. The results of such an experiment are shown in Fig. 7A, where ³⁵S-labeled TBP, CBP, and cyclin T could efficiently bind to wild-type but not acetylated 42–50 peptide. A similar experiment was performed using a purified HeLa core-Pol II fraction (Inostroza *et al.*, 1994; Piras *et al.*, 1994), followed by Western blot for Pol II. Results from Western blots indicated that wild-type and not acetylated peptide was capable of binding to core-Pol II. In an attempt to find substrates that could bind to acetylated peptide, we performed a series of binding assays followed by excision of bound peptides from gels and subjected them to mass spectrometry. The initial material for pull-down assays was whole HIV-1 virus (pDH125, a generous gift from M. Chow and M. Martin) reconstituted *in vitro* with all four histones. The bound complexes were washed with 150 mM salt and 0.5% NP-40, run on SDS-PAGE, and silver-stained, and peptides were excised and subjected to mass spectrometry. The results of such an experiment are shown in Fig. 7B, where acetylated Tat peptide was able to bind to core histones. It is interesting to note that core histones in the absence of DNA did not bind to acetylated Tat, indicating that a fixed conformation of nucleosome is required for Tat to bind to core histones. Therefore, these data collectively suggest that acetylated Tat may leave the initiation complex behind and retain its binding to nucleosomal DNA.

DISCUSSION

In recent years it has become apparent that non-DNA-binding transcriptional coactivators, such as p300 and CBP, that were previously thought to function primarily as "bridging" proteins between DNA-bound transcription factors and the basal transcription complex play a critical regulatory role as integrators of diverse signaling pathways in the selective induction of gene expression. Many examples of such phenomena are exemplified by CBP/p300's interaction with an array of transcription factors including sequence-specific DNA-binding proteins, such as the NF- κ B CREB, or activator protein 1 (AP-1) family members, that interact with the promoter and act as

FIG. 5. Mutation of the lysine residues at position 50 and 51 of Tat and their reduced LTR transactivation activity. (A) CAT assays were performed from lysates of transfected CEM (12D7) cells with LTR-CAT (3 μ g) and varying concentrations of CMV-Tat mutants (1 and 5 μ g). Lanes 1 and 2 represent basal transcription of LTR-CAT and Tat (1 μ g) activated transcription, respectively. Lanes 3–8 show transfection of single- or double-mutant Tat constructs into CEM cells. The mutants were pcTat (K50A, lanes 3, and 4), pcTat (K51A, lanes 5 and 6), and pcTat (K50A/K51A, lanes 7 and 8). Cells were transfected by the electroporation method and processed for CAT assay 24 h later. The percentage of CAT conversion is indicated at the bottom of each lane. (B) Cotransfection of pcTat (K50A/K51A) alone or with CBP (2.5 μ g) in CEM cells is shown in lanes 4 and 5, respectively. Lanes 1 and 6 serve as negative controls. Titration of either pcTat (K50A/K51A) or CBP plasmids showed similar results, where CBP was not able to rescue the Tat mutant construct (data not shown). (C) Transfection of various proteins along with LTR-CAT reporter into CEM cells. CEM cells were grown to log phase of growth and transfected with purified synthesized TAT 72 proteins of wild type, K 50/51 substituted with R, or K 50/51 substituted with A. A total of 3 μ g of LTR-CAT DNA along with 0.5 μ g of TAT proteins was electroporated into CEM cells (at 230 V). Extracts were analyzed 24 h after transfection for the presence of CAT enzyme.

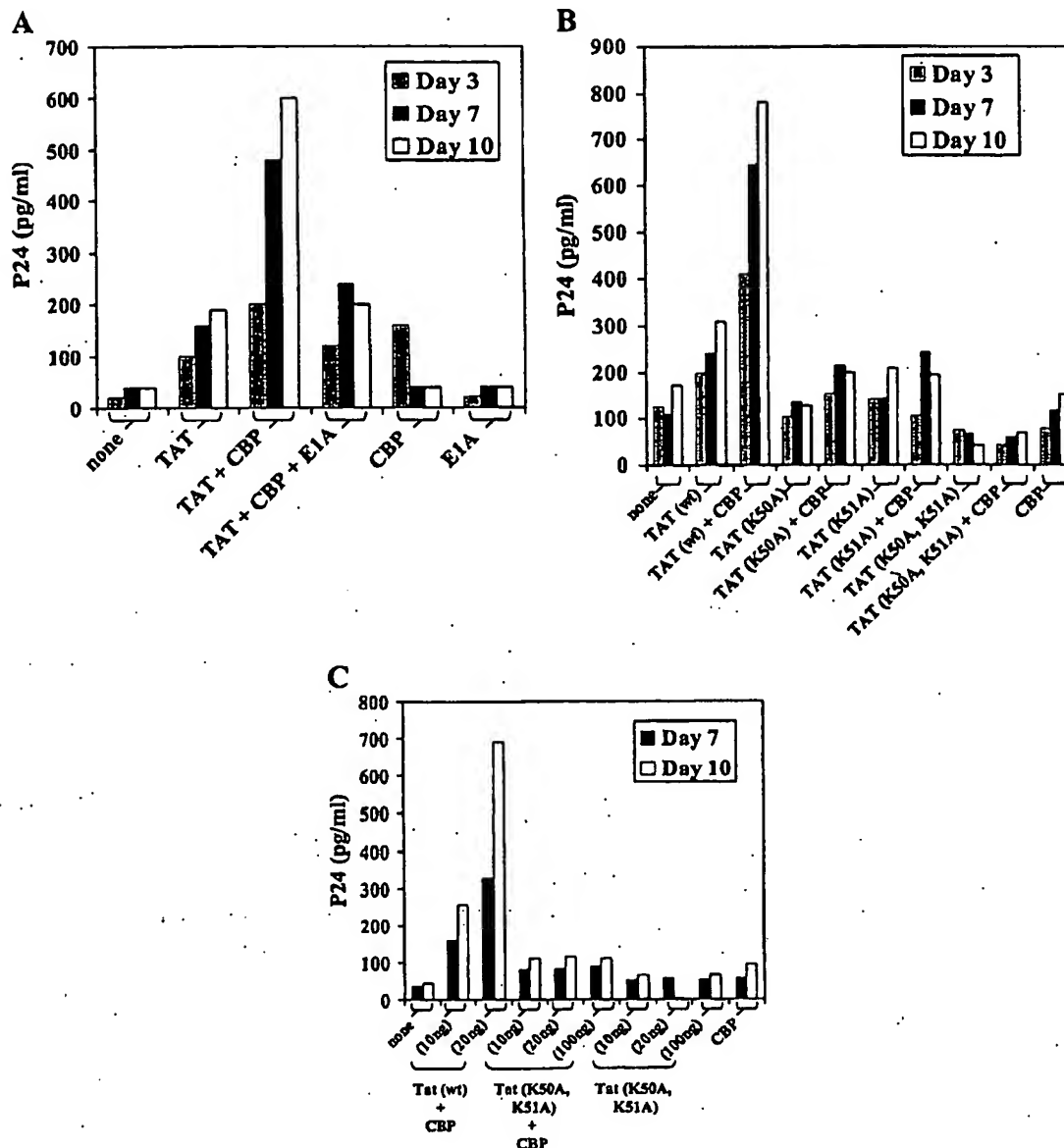


FIG. 6. Both lysines K50 and K51 of Tat play an important role in activation of the proviral integrated HIV-1 DNA. HLM1 cell is a HeLa-derived cell line that contains a wild-type integrated copy of the HIV-1 proviral genome except for the Tat open reading frame. Therefore, HLM1 cells are negative for virus particle production unless they are provided with cytokine signals, such as TNF, or ectopic addition of Tat, as determined by the presence of p24 gag antigen in the supernatant. (A) Coactivation of integrated HIV-1 provirus by Tat and CBP. HLM1 cells were transfected alone with Tat (50 ng), CMV-CBP (6 μ g), E1A (6 μ g), or in combination with each other by the CaPO₄ precipitation method. The p24 gag antigen ELISA was performed from supernatants obtained at days 3, 7, and 10 after transfection. It is interesting to note that cotransfection of Tat plus CBP increased the production of HIV-1 particles in HLM1 cells and E1A was able to reverse the CBP effect, presumably by binding to CBP and inhibiting the HAT activity. (B) HLM1 cells were transfected either alone with wild-type Tat and mutant Tat clones (single-mutants K50A and K51A and double-mutant K50A/K51A) or with CBP into HLM1 cells and p24 gag antigen measured at various time points. (C) Titration of double-mutant Tat (K50A/K51A) with CBP performed in HLM1 cells. Varying concentrations of the Tat double-mutant were transfected either alone or with 6 μ g of CBP. Supernatants were collected at days 7 and 10 for the p24 gag ELISA assay. All experiments in A, B, and C were performed twice.

either enhancers or repressors of gene expression during cellular activation. Members of the p300 and CBP family also appear to be present only in higher eukaryotic cells including *Caenorhabditis elegans* and *Drosophila*, and not in the yeast *Saccharomyces cerevisiae*. Thus, p300/CBP-like proteins are likely confined to multicellular organisms where they may fulfill specific functions required for proper growth and development.

Many viruses have evolved mechanisms to control both viral and host transcriptional machinery through CBP/p300. Generic virus infection of cells results in a dramatic hyperacetylation of histones H3 and H4 by CBP/p300, which is localized to target cellular promoters such as IFN- β promoter (Parekh and Maniatis, 1999). Also, both DNA and RNA viruses have evolved means to control CBP/p300 in both activation and repression. For

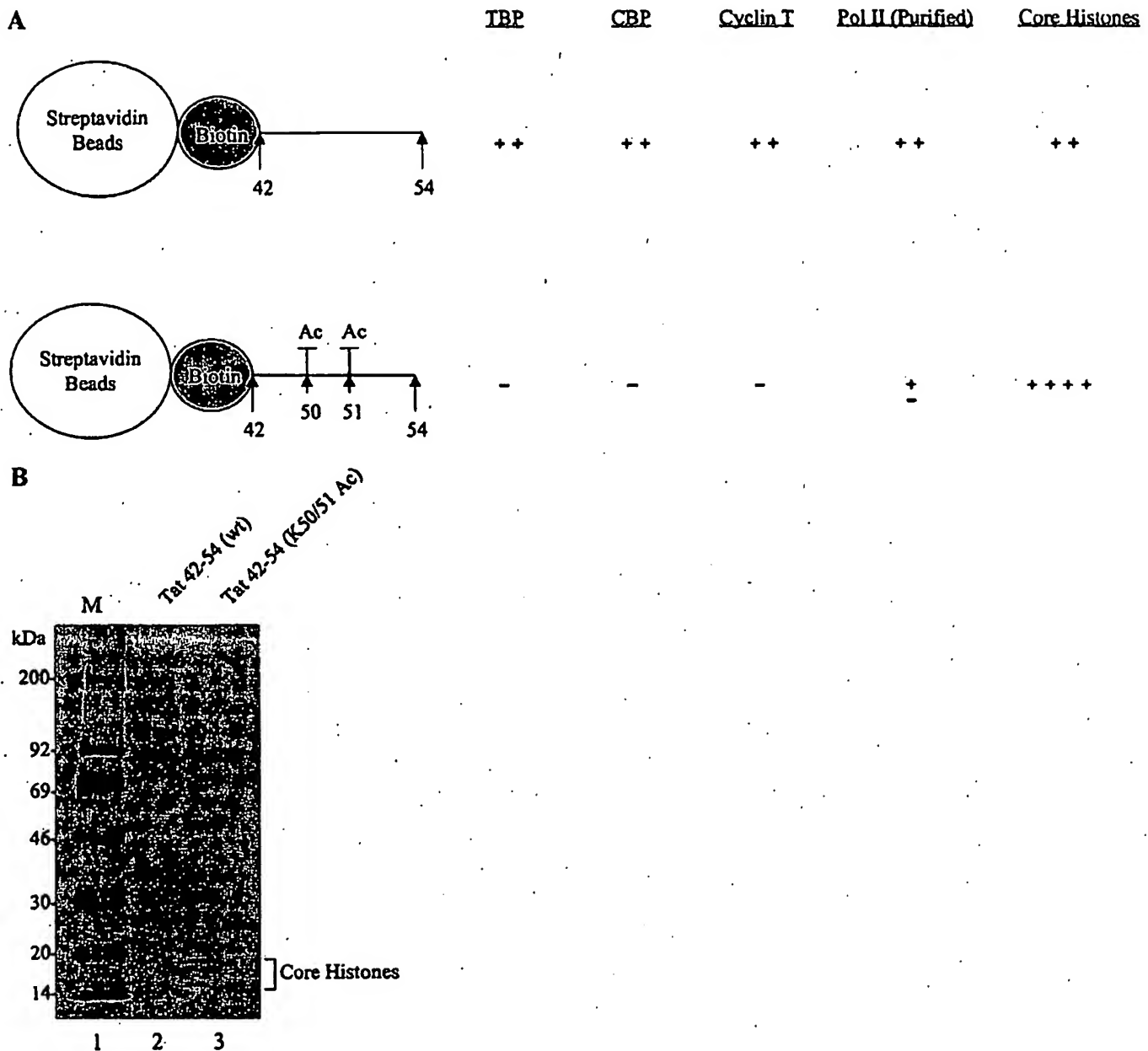


FIG. 7. Binding of acetylated Tat to various transcription factors. (A) Schematic representation of the pull-down experiment and the results of Western blots of the pull-down components with anti-TBP, CBP, cyclin T, and Pol II. The synthesized Tat peptides (42-54) with or without acetylated lysines at positions 50 and 51 were labeled with biotin at the N-terminus. The peptides were incubated with *in vitro* translated, [35 S]methionine/cysteine-labeled TBP, CBP, cyclin T, and purified core-HeLa Pol II. Streptavidin-agarose beads were used to pull down the peptide-associated complexes. The pull-down complexes were washed three times and separated on the 4-20% SDS-PAGE gel. For Western blots they were transferred to PVDF membranes and blotted with the antibodies against TBP, CBP, cyclin T, and Pol II. Pull-down experiments with 35 S-labeled TBP, CBP, and cyclin T were also performed to confirm the Western blot data. (B) Pull-down complexes from chromatin assembled HIV-1 DNA with the wild-type unacetylated Tat peptide (42-54, lane 2) or double-acetylated Tat peptide (42-54) at positions 50 and 51 lysines (lane 3) were separated on SDS-PAGE, stained with Coomassie blue, excised, subjected to digestion for trypsin digest, and analyzed by mass spectrometry. Western blots for histones H2A, H2B, H3, and H4 were also performed to confirm the mass spectrometry analysis (data not shown).

instance, the oncogenic human papillomavirus type 16 (HPV-16) E6 binds to three regions (C/H1, C/H3, and the C-terminus) of both CBP and p300. Interestingly, HPV-16 E6 inhibits the intrinsic transcriptional activity of CBP/p300 and decreases the ability of p300 to activate p53- and NF- κ B-responsive promoter elements (Patel *et al.*, 1999). Similarly, human herpesvirus 8/Kaposi sarcoma-

associated virus IRF 1 protein also targets the carboxy-terminal region (aa 1623 to 2414) of the transcriptional coactivator p300 (Burysek *et al.*, 1999). RNA retroviruses such as MMTV, HTLV-I, and HIV have also been noted for regulating p300/CBP. CBP suppresses the responsiveness of the mouse mammary tumor virus (MMTV) promoter to dexamethasone in a dose-dependent fashion

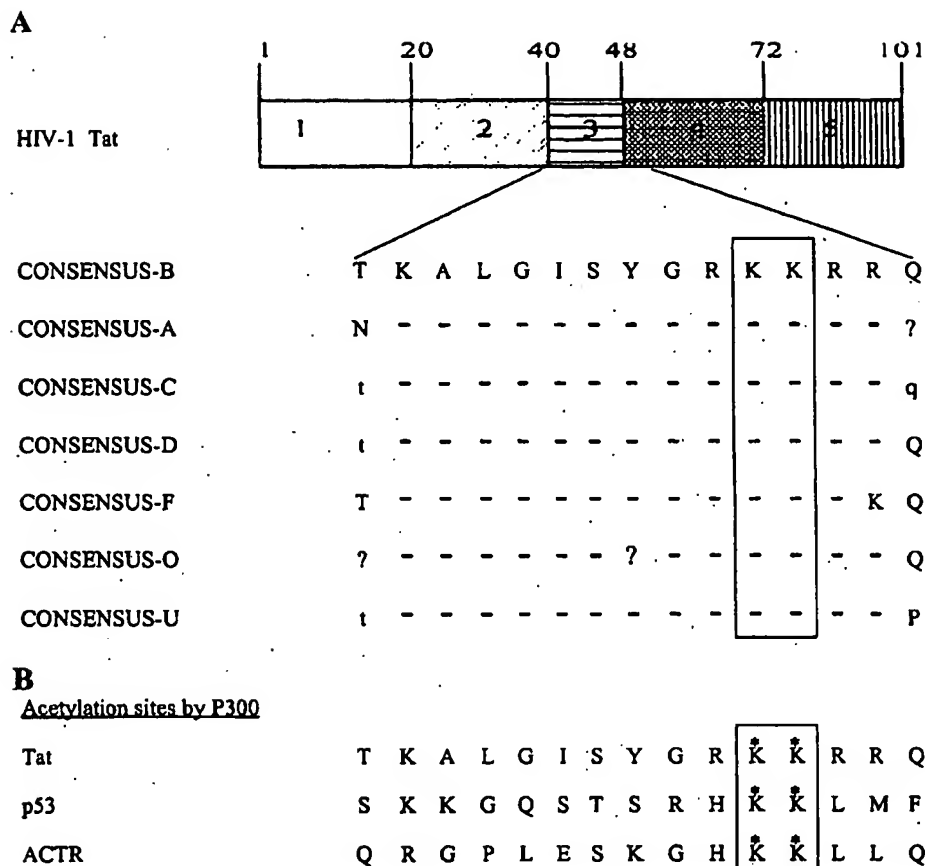


FIG. 8. A general scheme of various Tat domains. (A) Conservation of the double-lysine motif of Tat among various HIV-1 clades. The Tat protein and peptides used in this study were of North American Clade B type. (B) The double-lysine motifs of Tat resemble those in p53 and ACTR proteins, which are also acetylated by CBP/p300 as indicated by asterisks.

(Kino *et al.*, 1999), as well as alleviating NFI-C-mediated repression of MMTV by ectopic expression of p300/CBP (Chaudhry *et al.*, 1999). Transcriptional activation of the HTLV-I sequences by Tax and CBP is induced by reinitiation of transcription (Kashanchi *et al.*, 1998), and cytokine and Tat regulation of HIV transcription requires binding of the p300 coactivator to the promoter region (Hottiger *et al.*, 1998; Hottiger and Nabel, 1998; Benkirane *et al.*, 1998; Marzio *et al.*, 1998; Kiernan *et al.*, 1999; Ott *et al.*, 1999).

CBP/p300 acetylates the core histones as well as nonhistone proteins such as the tumor suppressor protein p53, the hematopoietic transcription factor GATA-1, and the basal transcription factors TFIIE and TFIIIF, although their biological functions are still not well understood. To test whether HIV-1 Tat could also be specifically acetylated by CBP/p300, we used the purified full-length Tat (1-86) protein and found that the labeling was completely dependent on the presence of the CBP/p300 HAT domain. The labeling was most efficient with purified Tat and not GST-Tat (1-101), GST-Tat (1-86), GST-Tat (1-72), or 17 other GST-Tat mutants.

When using Tat peptides to pinpoint the amino acid residues important for acetylation, we found that the both

lysines in the highly conserved region (⁴⁹RKKRRQ⁵⁴) of the basic RNA-binding motif of Tat were acetylated by CBP/p300 (see Fig. 8). Importantly, upon transfection of K50A or K51A Tat vectors with reporter HIV-LTR CAT, we observed a slight drop in transcriptional activity but a more pronounced drop in transactivation with the double-mutant K50A and K51A. This suggested that both lysines together are important in the transient transfection assays. However, when using HLM1 cells, containing integrated virus, we observed that both point mutations at position 50 and 51 were equally deleterious in activation of the integrated chromatinized HIV-1 DNA and that ectopic expression of the CBP could not activate either of the 50 or 51 mutants in these cells. Therefore, we believe that both lysines are equally important for *in vivo* activation of the latent virus.

When examining for the effect of wild-type or acetylated Tat on RNA binding, we found that the acetylated Tat peptide or protein was not able to bind to TAR RNA. This is in marked contrast to other acetylated proteins such as p53, where acetylation increases the DNA-binding activity (Gu and Roeder, 1997). More interestingly, when performing competition experiments with wild-type, acetylated, or alanine-substituted Tat peptides in

the same reaction, we observed that wild-type 41–54 but not other derivatives was able to enhance the binding of CBP to Tat as well as other basal factors such as TFIIB and TBP. This intriguing result indicates that CBP/p300 family members are subject to conformational change upon binding to viral and possibly cellular activators. In support of the change of conformation hypothesis, we have recently obtained preliminary data suggesting that *in vitro* translated CBP in presence of wild-type but not acetylated Tat peptide is susceptible to endoprotease Glu-C digestion and not to other nucleases such as trypsin, endonuclease Asp-N, or Lys-C proteases.

CBP/p300's ability to acetylate Tat has recently been examined in a chromatin reconstitution experiment. When using purified basal transcription factors NF- κ B, SP1, and cdk9/cyclin T in an *in vitro* transcription reaction, where the HIV-1 DNA is chromatinized, we observed no activated transcription *in vitro*, indicating that the mere presence of cdk9/cyclinT is not sufficient to drive RNA pol II through chromatin DNA. However, only in the presence of added minimal p300 HAT domain or wild-type p300 do we find activated transcription on HIV-1 DNA (Deng, unpublished data). Therefore, the role of the cdk9/cyclinT complex in HIV-1 transcription may be to phosphorylate the CTD of RNA pol II and that of p300 may be to acetylate the natural core histones on the HIV-1 genome. To this end, we have mapped the promoter region (–500 to +200) of 26 different HIV-1 clade isolates ranging from subtypes B to O and have observed that all viral isolates have chromatinized DNA *in vivo*, further indicating that the HIV-1 B clade that we have used in this study (in HLM1 cells) was not the only HIV-1 chromatinized template *in vivo*. Finally, our results show that the acetylated Tat decreases Tat's ability to bind the TAR RNA element, as well as to bind basal factors TBP and core-Pol II, but increases the efficiency of binding to core histones and only when assembled as a nucleosomal HIV-1 DNA. This notion may provide a mechanism of how Tat is able to leave the initiation complex behind and to facilitate chromatin modification or remodeling downstream of the transcription initiation site, perhaps by aiding in disruption of the nuc-1 nucleosome. El Kharroubi *et al.* (1998) demonstrated that expression of a functional Tat could alter the chromatin structure downstream of the HIV-1 promoter and that the binding of Tat to TAR (as occurs with Tat K41T) failed to induce chromatin remodeling. Recently, using *in vitro* reconstituted chromatin templates, we found that Tat-p300 interaction increases the acetylation of a nucleosomal histone. Such enhancement of histone acetylation may be due to the finding that the acetylated Tat binds with higher efficiency to nucleosomal DNA and changes the conformation, resulting in the accessibility of histone tails to p300. Acetylation of histones may flag other proteins needed for disruption of nuc-1 and subsequent transcription. Future experiments will determine the nu-

cleosomal position(s) that is affected by acetylated Tat on whole HIV-1 DNA.

MATERIALS AND METHODS

Cell culture and labeling

Log-phase CEM (12D7) T-lymphocytes (Kashanchi *et al.*, 1992, 1994a, 1994b) were grown in culture at 37°C up to 1×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum treated with a mixture of 1% streptomycin, penicillin, and 1% L-Glutamine (Gibco BRL). HLM-1 cells (AIDS Research and Reference Reagent Program, Catalog No. 2029) were derived from HeLa-T4⁺ cells integrated with one copy of the HIV-1 genome containing a Tat-defective mutation. The mutation was introduced as a triple termination linker at the first AUG of the Tat gene (Sadaie and Hager, 1994). HLM-1 cells are negative for virus particle production, but can be induced to express noninfectious HIV-1 particles after transfection with Tat cDNA or mitogens such as TNF- α or sodium butyrate. HLM1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 100 μ g/ml of G418, plus 1% streptomycin, penicillin, and 1% L-glutamine (Gibco BRL). These cells were always grown to 75% confluency before transfection or passages.

Labeling experiments were performed on log-phase 75% confluent HeLa/eTat or control HeLa/pcep4 cells (Kashanchi *et al.*, 2000). Cells were pulsed for 3 h with [³H]acetate (0.4 mCi, ICN) in complete DMEM plus hygromycin. Cells were also incubated overnight with [³⁵S]methionine/cysteine (0.2 mCi/ml, NEN) in methionine/cysteine-free medium and 10% dialyzed FCS. Nuclear lysates were prepared in 1 ml of lysis buffer (300 mM NaCl, 0.1% NP-40, 10 mM NaH₂PO₄, 1 mM EDTA, pH 8, 1 mM DTT, 50 mM sodium pyrophosphate, 10 mM NaF, 8 mM sodium butyrate), and immunoprecipitations were performed on 0.5 mg protein extract. Tat was detected using immunoprecipitation from cellular lysates with monoclonal 12CA5 Ab cross-linked to beads for 3 h and eluted with a 100-fold excess of influenza peptide for 8 h at 4°C. The sequence of peptide used for elution was as follows: ¹YPYDVPDYASL⁹. Four independent [³H]acetate and [³⁵S]methionine/cysteine labeling experiments were performed with similar results.

Lymphocyte transfection

Lymphocytes (CEM, 12D7) were grown to mid-log phase of growth and were processed for electroporation according to a previously published procedure (Kashanchi *et al.*, 1992). Only one modification was introduced, where cells were electroporated at 230 V and plated in 10 ml of complete RPMI 1640 medium for 18 h prior to harvest and CAT assay.

Transfection and HIV-1 detection of HLM₁ cells

HLM₁ cells were propagated in DMEM (containing 100 μ g/ml of G418) and transfected with the plasmid DNAs including Tat, mutant Tat, and CBP using the calcium phosphate method. The transfected cells were washed after 4 h and fresh complete DMEM with 10% fetal bovine was added for the remainder of the experiment. The p24 gag antigen was detected with a standard ELISA kit (Abbott) using the supernatants of transfected cells at days 3, 7, and 10.

Plasmids

HIV-LTR-CAT reporter and eukaryotic Tat expression vectors (pcTat) have been described previously (Hauber *et al.*, 1989; Kashanchi *et al.*, 1994b). Mutants of the lysine residue at position 50 and/or 51 of the Tat expression plasmid were constructed from pcTat, by replacing the *Eco*NI fragment with synthesized mutated oligo adaptor. The following top strands for each mutated construct are indicated: for K50A, 5'-GGCAGG-GCGAAGCGGAGACAGCGACGAAGACCTCC3'; for K51A, 5'-GGCAGGAAGGCGCGGAGACAGCGACGAAGACCTCC3'; and for K50/A+K51/A, 5'-GGCAGGGCGCGCGGAGAC-AGCGACGAAGACCTCC3'. Eukaryotic expression vectors for CBP E1A have previously been reported (Chakravarti *et al.*, 1999). GST-p300 (HAT) from aa 1197 to 1735 was PCR amplified from a p300 B fragment as well as the p300 C fragment and subcloned into pGEX (more details of subcloning will be provided upon request). The resulting recombinant vector was transformed into *E. coli* DH5a and were grown overnight in 10 ml of LB with 100 μ g/ml of ampicillin. A 500-ml LB + ampicillin flask was inoculated with the overnight culture and was grown for 4 h at 37°C. Isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 0.1 mM to induce fusion protein expression, and the culture was switched to 30°C for an additional 4 h. Cells were collected by centrifugation in a GSA rotor at 5800 *g* for 10 min at 4°C. For sonication, the bacterial pellet was resuspended in 25 ml of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and was sonicated (Branson) for 35 pulses at the 3.5 microprobe setting. The resulting mixture was centrifuged in a GSA rotor at 5800 *g* for 10 min at 4°C. A second centrifugation in a SS-34 rotor at 23,500 *g* for 20 min at 4°C clarified the extract of remaining debris. GST-fusion proteins were bound to agarose beads overnight, washed the next day, and run on 4–20% SDS-PAGE for both quality and quantity prior to use in HAT assays.

Baculovirus expression and protein purifications

Sf9 cells were grown to mid-log phase in HyClone HyQ CCM-3 serum-free medium utilizing spinner flask culture methods. For p300 infection, the cells were infected with 11 ml of p300 FLAG virus at 2.0×10^6 cells/ml and then

allowed to incubate at 10 rpm for approximately 1 h. After this initial incubation/infection time the spinner plates were turned up to 70 rpm for the remainder of the incubation. The cells were collected via centrifugation 48 h after infection. The cell culture normally yielded approximately 2 ml of PCV from 500 ml of original culture volume, and the cell pellet was processed for further purification. Samples were lysed with lysis buffer, containing 50 mM Tris-Cl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF (phosphotyrosine phosphatase inhibitor), 1 mM DTT, and 1 mM PMSF, and processed with 12CA5 monoclonal antibodies for further purifications.

Histone acetyltransferase assay

HAT assays were performed as 30- μ l total reactions at 37°C for 60 min in buffer containing 1 μ l of purified GST-p300 HAT (1 mg/ml), 200–400 ng substrate proteins or peptides, and 1 μ g of histones H2A, H2B, H3, and H4 in 20 mM HEPES-NaOH, pH 7.4, 1 mM dithiothreitol, 10 mM sodium butyric acid, and 1 μ l of [¹⁴C]acetyl-Coenzyme A (65 mCi/mmol, ICN). Proteins and peptides were resolved on 4–20% or 15% SDS-PAGE. Gels were dried and exposed to a PhosphorImager cassette.

Preparation of TAR RNA and RNA-binding experiments

The plasmid pT7 was constructed from pU3R-III containing a T7 promoter at nucleotide +1 of HIV (Gunnery *et al.*, 1990). pT7 was linearized at nucleotide +82 by digestion with *Hind*III and transcribed using T7 RNA polymerase (Promega). TAR RNA was labeled with [α -³²P]UTP and was subjected to electrophoresis in a 10% polyacrylamide gel. The major radioactive RNA band was eluted and extracted with phenol/chloroform and precipitated with ethanol.

Gel mobility shift reaction (16- μ l final reaction volume) was carried out in binding buffer (10 mM HEPES, pH 7.3; 100 mM KCl; 1 mM MgCl₂; 0.5 mM EDTA; 1 mM DTT; and 10% glycerol) and contained 3 ng of labeled TAR RNA as well as 200 ng tRNA as nonspecific competitor. Reactions were incubated for 30 min at room temperature and RNA-bound complexes were separated on a prerun 6% DNA retardation gel (Novex) containing 0.5 \times TBE buffer, at 7 W for 2.5 h at 4°C.

Peptide synthesis

The biotinylated peptides were prepared on a PAL-PEG-polystyrene resin by continuous-flow solid-phase synthesis on a PerSeptive Biosystems Pioneer synthesizer (Framingham, MA) using HBTU-activated Fmoc amino acids. Side chain protection was as follows: Arg (Pmc), Gln (Trt), Lys (Dde), Ser, and Tyr (Bu^t). Peptide assembly was concluded by N^α-acylation with HBTU-activated biotin. The resin-bound peptide was then

treated with 3% hydrazine in DMF for 20 min to selectively remove the Dde groups from the side chain of Lys. The resin was then divided into two equal portions and one-half was subjected to a 1-h treatment with an excess of acetic anhydride in the presence of an equivalent amount of base to acetylate the resulting free N^ε groups. Both peptides were then separately cleaved from the solid support and simultaneously the remaining side chain was deprotected by reaction with trifluoroacetic acid in the presence of scavengers. Peptide purification was achieved by conventional reverse-phase HPLC on Vydac C18 (Hesperia, CA) in an overall yield of 25–30% based on starting resins. The purity of the two peptides was confirmed by analytical reverse-phase HPLC, capillary zone electrophoresis, and matrix-assisted laser desorption time of flight mass spectrometry. For the non-acetylated peptide, we found MH⁺ 1757.5 (calc. MH⁺ 1757.1). For the acetylated peptide, we found MH⁺ 1840.9 (calc. MH⁺ 1840.1).

Synthesis of acetylated peptides at positions 50, 51, and 50+51 was carried out on the ABI 433A Peptide Synthesizer (PE Biosystems, Foster City, CA) using Fastmoc chemistry with N^ε-acetyl-L-lysine, which was purchased from Novabiochem (San Diego, CA). After cleavage and deprotection, the peptides were purified by HPLC (Dionex, Sunnyvale, CA) using an acetonitrile gradient on a C18 reverse-phase column (Pharmacia, Piscataway, NJ). The amount of protein was determined by Bio-Rad protein assay as well as by running small aliquots on 4–20% SDS-PAGE followed by silver staining.

Streptavidin bead pull-down assay

Synthesized Tat (42–54) peptides, labeled with biotin at the N-terminus, and with or without an acetyl group at lysines 50 and 51, were used in the pull-down assays. The biotin-labeled Tat peptides were incubated with the cell extracts in TNE₅₀ buffer (100 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; 0.1% NP 40) at 4°C overnight. Streptavidin beads (Boehringer Mannheim) were added to the mixture and incubated for 2 h at 4°C. The beads were washed three times with TNE₁₅₀ (100 mM Tris-HCl, pH 7.5, 150 mM NaCl; 1 mM EDTA; 0.1% NP-40). The bound proteins were separated on 4–20% SDS-PAGE and subjected to Western blotting with antibodies against TBP, CBP, cyclin T, and RNA polymerase II (Santa Cruz Inc.; SC-900 (C21) for Pol II, Sc-1211 (451) for CBP, Sc-204 (N12) for TBP, and cyclin T (a generous gift from M. Mathews).

Nucleosome reconstitution by salt dialysis

The core histones were purified from HeLa cells by the method of Simon and Felsenfeld (1979). Chromatins were prepared from high-molecular-weight DNA and plain and purified core histones by dialysis from 1 M NaCl (Imbalzano, 1998; Stein, 1989). Ten micrograms of

plasmid DNA of pDH125 (whole HIV-1 genome, a generous gift from M. Cho and M. Martin, NIAID/NIH) was mixed with 5 μl of 5 M NaCl and 2 μl of 10× reconstitution buffer (0.15 M Tris-HCl, pH 7.5; 1 mM DTT; 2 mM EDTA) by pipetting up and down repeatedly. Later, 15 μg of core histones was added in a total volume of 20 μl; the volume was adjusted by adding ddH₂O. Samples were gently flicked in the tube to mix and incubated at 37°C for 20 min. Sequential dilution was carried out by adding 10 μl of 1× reconstitution buffer every 10 min, for 3 h at 37°C. At each time point, samples were mixed by pipetting up and down. An aliquot was run on agarose gels to ensure proper assembly prior to each experiment.

In-gel digestion, mass spectrometry, and protein identification

The in-gel digestion was performed based on a procedure previously described by Fernandez *et al.* (1998). The gel bands of interest were excised from SDS-PAGE and digested with 0.2 μg of trypsin (Promega modified sequencing grade trypsin). The digests were desalted using C₁₈ ZipTips (Millipore) according to the manufacturer's protocol. A 1-μl aliquot of sample was taken for peptide mass mapping on a PerSeptive Biosystem DEPRO MALDI-TOF Mass Spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix. Analysis was performed in the linear delayed-extraction mode, with external calibration. Protein identification by mass mapping was performed through the ProFound Web site located at Rockefeller University (prowl.rockefeller.edu).

ACKNOWLEDGMENTS

We thank Ebony Brooks and Carolyn Eadie for assistance in preparing the manuscript. We also acknowledge Ms. Nicola Dawson (Howard Florey Institute) for preparing the biotinylated Tat peptides. We thank Angela Huber (Research Associate) at National Cell Culture Center (NIH funded) for preparing the Baculovirus stocks and cultures. This work was supported by NIH Grants AI44357 and AI43894 and by a UMDNJ Foundation Grant to F.K.

REFERENCES

- Adams, M., Sharmeen, L., Kimpton, J., Romeo, J. M., Garcia, J. V., Peterlin, B. M., Groudine, M., and Emerman, M. (1994). Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. *Proc. Natl. Acad. Sci. USA* 91, 3862–3866.
- Ait-Si-Ali, S., Ramirez, S., Barre, F. X., Dkhissi, F., Magnaghi-Jaulin, L., Girault, J. A., Robin, P., Knibiehler, M., Pritchard, L. L., Ducommun, B., Trouche, D., and Harel-Bellan, A. (1998). Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. *Nature* 396, 184–186.
- Bannister, A. J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. *Nature* 384, 641–643.
- Benkirane, M., Chun, R. F., Xiao, H., Ogryzko, V. V., Howard, B. H., Nakatani, Y., and Jeang, K. T. (1998). Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. *J. Biol. Chem.* 273, 24898–24905.
- Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., and Cullen, B. R. (1999).

- Recruitment of cyclin T1/P-TEFb to an HIV type 1 long terminal repeat promoter proximal RNA target is both necessary and sufficient for full activation of transcription. *Proc. Natl. Acad. Sci. USA* 96, 7791-7796.
- Blanco, J. C., Minucci, S., Lu, J., Yang, X. J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998). The histone acetylase PCAF is a nuclear receptor coactivator. *Genes Dev.* 12, 1638-1651.
- Burysek, L., Yeow, W. S., Lubyova, B., Kellum, M., Schafer, S. L., Huang, Y. Q., and Pitha, P. M. (1999). Functional analysis of human herpesvirus 8-encoded viral interferon regulatory factor 1 and its association with cellular interferon regulatory factors and p300. *J. Virol.* 73, 7334-7342.
- Chakravarti, D., Ogryzko, V., Kao, H. Y., Nash, A., Chen, H., Nakatani, Y., and Evans, R. M. (1999). A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity. *Cell* 96, 393-403.
- Chaudhry, A. Z., Vitullo, A. D., and Gronostajski, R. M. (1999). Nuclear factor I-mediated repression of the mouse mammary tumor virus promoter is abrogated by the coactivators p300/CBP and SRC-1. *J. Biol. Chem.* 274, 7072-7081.
- Chen, D., Fong, Y., and Zhou, Q. (1999a). Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-1 transcription. *Proc. Natl. Acad. Sci. USA* 96, 2728-2733.
- Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999b). Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* 98, 675-686.
- Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 90, 569-580.
- Chivla, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* 365, 855-859.
- Creaven, M., Hans, F., Mutskov, V., Col, E., Caron, C., Dimitrov, S., and Khochbin, S. (1999). Control of the histone-acetyltransferase activity of Tip60 by the HIV-1 transactivator protein, Tat. *Biochemistry* 38, 8826-8830.
- Cujec, T. P., Cho, H., Maldonado, E., Meyer, J., Reinberg, D., and Peterlin, B. M. (1997). The human immunodeficiency virus transactivator Tat interacts with the RNA polymerase II holoenzyme. *Mol. Cell. Biol.* 17, 1817-1823.
- Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994). Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.* 8, 869-884.
- El Kharroubi, A., Piras, G., Zensen, R., and Martin, M. A. (1998). Transcriptional activation of the integrated chromatin-associated human immunodeficiency virus type 1 promoter. *Mol. Cell. Biol.* 18, 2535-2544.
- Felzien, L. K., Farrell, S., Betts, J. C., Mosavin, R., and Nabel, G. J. (1999). Specificity of cyclin E-Cdk2, TFIIB, and E1A interactions with a common domain of the p300 coactivator. *Mol. Cell. Biol.* 19, 4241-4246.
- Fernandez, J., Gharahdaghi, F., and Mische, S. M. (1998). Routine identification of proteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or polyvinylidene difluoride membranes using matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). *Electrophoresis* 19, 1036-1045.
- Fujinaga, K., Cujec, T. P., Peng, J., Garriga, J., Price, D. H., Grana, X., and Peterlin, B. M. (1998). The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1, and Tat. *J. Virol.* 72, 7154-7159.
- Garber, M. E., Wei, P., Kewal Ramani, V. N., Mayall, T. P., Herrmann, C. H., Rice, A. P., Littman, D. R., and Jones, K. A. (1998). The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. *Genes Dev.* 22, 3512-3527.
- Garcia-Martinez, L. F., Mavankal, G., Neveu, J. M., Lane, W. S., Ivanov, D., and Gaynor, R. B. (1997). Purification of a Tat-associated kinase reveals a TFIIB complex that modulates HIV-1 transcription. *EMBO J.* 16, 2836-2850.
- Garriga, J., Peng, J., Parrenò, M., Price, D. H., Henderson, E. E., and Grana, X. (1998). Upregulation of cyclin T1/CDK9 complexes during T cell activation. *Oncogene* 24, 3093-3102.
- Goldman, P. S., Tran, V. K., and Goodman, R. H. (1997). The multifunctional role of the co-activator CBP in transcriptional regulation. *Rec. Prog. Horm. Res.* 52, 103-120.
- Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90, 595-606.
- Gunnery, S., Rice, A. P., Robertson, H. D., and Mathews, M. B. (1990). Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase. *Proc. Natl. Acad. Sci. USA* 87, 8687-8691.
- Hauber, J., Malim, M. H., and Cullen, B. R. (1989). Mutational analysis of the conserved basic domain of human immunodeficiency virus tat protein. *J. Virol.* 63, 1181-1187.
- Henderson, E. E., Tsygankov, A. Y., Merlo, J. J., Romano, G., and Guan, M. (1999). Altered replication of human immunodeficiency virus type 1 (HIV-1) in T cell lines retrovirally transduced to express herpesvirus saimiri proteins StpC and/or Tip. *Virology* 264, 125-133.
- Herrmann, C. H., and Rice, A. P. (1995). Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: Candidate for a Tat cofactor. *J. Virol.* 69, 1612-1620.
- Hottiger, M. O., and Nabel, G. J. (1998). Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein. *J. Virol.* 72, 8252-8256.
- Hottiger, M. O., Felzien, L. K., and Nabel, G. J. (1998). Modulation of cytokine-induced HIV gene expression by competitive binding of transcription factors to the coactivator p300. *EMBO J.* 17, 3124-3134.
- Imbalzano, A. N. (1998). SWI/SNF complexes and facilitation of TATA binding protein-nucleosome interactions. *Methods* 15, 303-314.
- Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., and Ge, H. (1997). Acetylation of general transcription factors by histone acetyltransferases. *Curr. Biol.* 7, 689-692.
- Inostroza, J., Flores, O., and Reinberg, D. (1994). Factors involved in specific transcription by mammalian RNA polymerase II. Purification and functional analysis of general transcription factor IIE. *J. Biol. Chem.* 269, 9304-9308.
- Isel, C., and Karn, J. (1999). Direct evidence that HIV-1 Tat stimulates RNA polymerase II carboxyl-terminal domain hyperphosphorylation during transcriptional elongation. *J. Mol. Biol.* 292, 929-941.
- Ivanov, D., Kwak, Y. T., Nee, E., Guo, J., Garcia-Martinez, L. F., and Gaynor, R. B. (1999). Cyclin T1 domains involved in complex formation with Tat and TAR RNA are critical for tat-activation. *J. Mol. Biol.* 292, 41-56.
- Jeang, K. T., Chun, R., Lin, N. H., Gatignol, A., Glabe, C. G., and Fan, H. (1993). In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor. *J. Virol.* 67, 6224-6233.
- Kamine, J., Elangovan, B., Subramanian, T., Coleman, D., and Chinnadurai, G. (1996). Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 tat transactivator. *Virology* 216, 357-366.
- Kao, S. Y., Calman, A. F., Luciw, P. A., and Peterlin, B. M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* 330, 489-493.
- Kashanchi, F., Duvall, J. F., and Brady, J. N. (1992). Electroporation of viral

- transactivator proteins into lymphocyte suspension cells. *Nucleic Acids Res.* 20, 4673-4674.
- Kashanchi, F., Piras, G., Radonovich, M. F., Duvall, J. F., Fattaey, A., Chiang, C. M., Roeder, R. G., and Brady, J. N. (1994a). Direct interaction of human TFIIID with the HIV-1 transactivator tat. *Nature* 367, 295-299.
- Kashanchi, F., Shibata, R., Ross, E. K., Brady, J. N., and Martin, M. A. (1994b). Second-site long terminal repeat (LTR) revertants of replication-defective human immunodeficiency virus: Effects of revertant TATA box motifs on virus infectivity, LTR-directed expression, in vitro RNA synthesis, and binding of basal transcription factors TFIIID and TFIIA. *J. Virol.* 68, 3298-3307.
- Kashanchi, F., Duvall, J. F., Kwok, R. P., Lundblad, J. R., Goodman, R. H., and Brady, J. N. (1998). The coactivator CBP stimulates human T-cell lymphotropic virus type I Tax transactivation in vitro. *J. Biol. Chem.* 273, 34646-34652.
- Kashanchi, F., Agbottah, E. T., Pise-Masison, C. A., Mahieux, R., Duvall, J., Kumar, A., and Brady, J. N. (2000). Cell cycle-regulated transcription by the human immunodeficiency virus type 1 Tat transactivator. *J. Virol.* 74, 652-660.
- Kiernan, R. E., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calomte, C., Bumy, A., Nakatani, Y., Jeang, K. T., Benkirane, M., and Van Lint, C. (1999). HIV-1 Tat transcriptional activity is regulated by acetylation. *EMBO J.* 18, 6106-6118.
- Kino, T., Nordeen, S. K., and Chrousos, G. P. (1999). Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300. *J. Steroid Biochem. Mol.* 70, 15-25.
- Lai, J. S., and Herr, W. (1992). Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. *Proc. Natl. Acad. Sci. USA* 89, 6958-6962.
- Lipinski, K. S., Fax, P., Wilker, B., Hennemann, H., Brockmann, D., and Esche, H. (1999). Differences in the interactions of oncogenic adenovirus 12 early region 1A and nononcogenic adenovirus 2 early region 1A with the cellular coactivators p300 and CBP. *Virology* 255, 94-105.
- Majello, B., Napolitano, G., and Lania, L. (1998). Recruitment of the TATA-binding protein to the HIV-1 promoter is a limiting step for Tat transactivation. *AIDS* 12, 1957-1964.
- Martinez-Balbas, M. A., Bannister, A. J., Martin, K., Haus-Seuffert, P., Meisterernst, M., and Kouzarides, T. (1998). The acetyltransferase activity of CBP stimulates transcription. *EMBO J.* 17, 2886-2893.
- Marzio, G., Tyagi, M., Gutierrez, M. I., and Giacca, M. (1998). HIV-1 tat transactivator recruits p300 and CREB-binding protein histone acetyltransferases to the viral promoter. *Proc. Natl. Acad. Sci. USA* 95, 13519-13524.
- Mavankal, G., Ignatius Ou, S. H., Oliver, H., Sigman, D., and Gaynor, R. B. (1996). Human immunodeficiency virus type 1 and 2 Tat proteins specifically interact with RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 93, 2089-2094.
- Napolitano, G., Licciardo, P., Gallo, P., Majello, B., Giordano, A., and Lania, L. (1999). The CDK9-associated cyclins T1 and T2 exert opposite effects on HIV-1 Tat activity. *AIDS* 13, 1453-1459.
- O'Keefe, B., Fong, Y., Chen, D., Zhou, S., and Zhou, Q. (2000). Requirement for a kinase-specific chaperone pathway in the production of a Cdk9/cyclin T1 heterodimer responsible for P-TEFb-mediated tat stimulation of HIV-1 transcription. *J. Biol. Chem.* 275, 279-287.
- Ott, M., Schnolzer, M., Garnica, J., Fischle, W., Emiliani, S., Rackwitz, H. R., and Verdin, E. (1999). Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity. *Curr. Biol.* 9, 1489-1492.
- Parada, C. A., and Roeder, R. G. (1996). Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain. *Nature* 384, 375-378.
- Parekh, B. S., and Maniatis, T. (1999). Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. *Mol. Cell* 3, 125-129.
- Patel, D., Huang, S. M., Baglia, L. A., and McCance, D. J. (1999). The E6 protein of human papillomavirus type 16 binds to and inhibits coactivation by CBP and p300. *EMBO J.* 18, 5061-5072.
- Piras, G., Kashanchi, F., Radonovich, M. F., Duvall, J. F., and Brady, J. N. (1994). Transcription of the human T-cell lymphotropic virus type I promoter by an alpha-amanitin-resistant polymerase. *J. Virol.* 68, 6170-6179.
- Ramanathan, Y., Reza, S. M., Young, T. M., Mathews, M. B., and Pe'ery, T. (1999). Human and rodent transcription elongation factor P-TEFb: Interactions with human immunodeficiency virus type 1 tat and carboxy-terminal domain substrate. *J. Virol.* 73, 5448-5458.
- Romano, G., Kasten, M., De Falco, G., Micheli, P., Khalili, K., and Giordano, A. (1999). Regulatory functions of Cdk9 and of cyclin T1 in HIV tat transactivation pathway gene expression. *J. Cell Biochem.* 75, 357-368.
- Sadale, M. R., and Hager, G. L. (1994). Induction of developmentally programmed cell death and activation of HIV by sodium butyrate. *Virology* 202, 513-518.
- Sang, N., Avantaggiati, M. L., and Giordano, A. (1997). Roles of p300, pocket proteins, and hTBP in E1A-mediated transcriptional regulation and inhibition of p53 transactivation activity. *J. Cell Biochem.* 66, 277-285.
- Simon, R. H., and Felsenfeld, G. (1979). A new procedure for purifying histone pairs H2A + H2B and H3 + H4 from chromatin using hydroxylapatite. *Nucleic Acids Res.* 6, 689-696.
- Shikama, N., Lyon, J., and La Thangue, N. B. (1997). The p300/CBP family: Integrating signals with transcription factors and chromatin. *Trends Cell Biol.* 7, 230-236.
- Stein, A. (1989). Reconstitution of chromatin from purified components. *Methods Enzymol.* 170, 585-603.
- Van Lint, C., Emiliani, S., Ott, M., and Verdin, E. (1996). Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *EMBO J.* 15, 1112-1120.
- Verdin, E. (1991). DNase I-hypersensitive sites are associated with both long terminal repeats and with the intragenic enhancer of integrated human immunodeficiency virus type 1. *J. Virol.* 65, 6790-6799.
- Veschambre, P., Simard, P., and Jalinot, P. (1995). Evidence for functional interaction between the HIV-1 Tat transactivator and the TATA box binding protein in vivo. *J. Mol. Biol.* 250, 169-180.
- Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 92, 451-462.
- Wimmer, J., Fujinaga, K., Taube, R., Cujec, T. P., Zhu, Y., Peng, J., Price, D. H., Peterlin, B. M. (1999). Interactions between Tat and TAR and human immunodeficiency virus replication are facilitated by human cyclin T1 but not cyclins T2a or T2b. *Virology* 255, 182-189.
- Yang, X., Herrmann, C. H., and Rice, A. P. (1996). The human immunodeficiency virus Tat proteins specifically associate with TAK in vivo and require the carboxyl-terminal domain of RNA polymerase II for function. *J. Virol.* 70, 4576-4584.
- Zhang, W., and Bieker, J. J. (1998). Acetylation and modulation of erythroid Kruppel-like factor (EKLF) activity by interaction with histone acetyltransferases. *Proc. Natl. Acad. Sci. USA* 95, 9855-9860.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record.**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.